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APPLICATION NUMBER: *60/540,557*
FILING DATE: *February 02, 2004*

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No.

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
1) Amir	ARAV	Tel Aviv

Additional inventors are being named on the 2nd *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

METHOD FOR LYOPHILIZATION OF BIOLOGICAL SAMPLES

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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OR

☐ Firm or Individual Name					
Address					
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Country		Telephone		Fax	

ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 31 with embedded drawings ☐ CD(s), Number

☐ Drawing(s) *Number of Sheets* ☑ Other (specify) transmittal, postcard

☐ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☑ Applicant claims small entity status. See 37 CFR 1.27.

☑ A check or money order is enclosed to cover the filing fees.

☑ The Director is herby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 14-0112

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☑ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

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Date February 2, 2004

REGISTRATION NO. 40,669 *(if appropriate)*

Docket Number: 25969

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Additional Page

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Docket Number 25969

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Number 2 of 2

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MAIL STOP PROVISIONAL PATENT APPLICATION
Attorney Docket No. 25969

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

ARAV et al.

Serial No. NOT YET ASSIGNED

Filed: February 2, 2004

For: **METHOD FOR LYOPHILIZATION OF BIOLOGICAL SAMPLES**

TRANSMITTAL LETTER

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Submitted herewith for filing in the U.S. Patent and Trademark Office is the following **PROVISIONAL APPLICATION:**

(1) Transmittal Letter
(2) Cover sheet for filing **Provisional Application**
(3) 31 page Provisional Application consisting of:
31 pages Textual Specification with embedded drawings,
0 pages of Claims,
0 page of the Abstract,
0 sheets of Drawings;
(4) Check No. 25969 $ 80.00 for filing fee as a small entity;
(5) Postcard for early notification of serial number.

The Commissioner is hereby authorized to charge any deficiency or credit any excess to Deposit Account No. 14-0112.

Respectfully submitted,
NATH & ASSOCIATES PLLC

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Form Page 1

Principal Investigator/Program Director (Last, First, Middle): . Arav, Amir

Fresh Red Blood Cells can be refrigerated for up to 42 days, after which they are discarded due to RBC recovery falling below 70%. Frozen RBC units are stored for up to 10 years but their use is restricted to emergencies because the intracellular cryopreservation agents (CPAs) used in the freezing must be washed out – a half hour process. This application presents research to freeze and thaw RBCs without CPAs and to achieve freeze-dried red blood cells that can be reconstituted by dilution with sterile water. These solutions may solve the long term RBC shortages in the USA and provide advantages for emergency medics.

When freezing blood cells the rate of cooling affects the morphology of the intracellular ice crystals; maximizing the survival rate of cells requires careful control of the freezing process. Conventional freezing devices involve lowering the temperature of the chamber in a controlled stepped manner. The thermal gradient within the sample is determined implicitly by the temperature of the chamber and the thermal conductivity of the materials of the sample, and is *not directly controllable*.

We **hypothesize that control of Erythrocyte Cooling Rate and the Interface Velocity can result in a high cell survival rate without CPAs, and a high survival rate of freeze dried RBCs**. By employing a novel freezing apparatus, "Multi-Temperature-Gradient" (MTG), we aim to overcome the above-mentioned problems and to enable the cryopreservation of large-volume samples. This freezing technology is based on **directional freezing**, in which the blood cell sample is transported in a customized container through a linear temperature gradient, which ensures precise control of the cooling rate and ice crystal front propagation. Thus, by controlling the velocity and morphology of the crystallization, mechanical damage caused during freezing of the blood cells is significantly reduced.

Having obtained encouraging results in preliminary studies, the specific aims of this project are to optimize cell survival and confirm post freezing functionality of RBCs both *in vitro* and *in vivo* (in a novel animal model to be developed at the outset); scale-up the freezing and freeze-drying technology to clinical-use volumes, then confirm earlier results on frozen / freeze-dried RBCs processed by the new devices.

PERFORMANCE SITE(S) *(organization, city, state)*

IMT LTD

CPA-FREE FREEZE-THAWED AND LYOPHILIZED RED BLOOD CELLS

PI: Dr Amir Arav

February 2004

RESEARCH PLAN

A Specific Aims

Long-term Objectives

When freezing blood cells the rate of cooling affects the morphology of the intercellular ice crystals[i]; morphologies such as closely packed needles kill cells by external mechanical damage (effect of AFP on blood cells – Carpenters)[ii iii iv]. Maximizing the survival rate of cells subjected to freezing and thawing requires careful control of the freezing process. The conventional freezing device involves lowering the temperature of th chamber in a controlled stepped manner. The conventional method is based on using multidirectional (equiaxial) heat transfer to achieve a rate of temperature change in the sample that depends on the thermal conductivity, the geometrical shape of the container and the biological material within it[v]. The thermal gradient within the sample is determined implicitly by the temperature of the chamber and the thermal conductivity of the materials of the sample, and is not directly controllable. This research project is based upon the **hypothesis that control of Erythrocyte Cooling Rate and the Interface Velocity can result in a high survival rate of the cells in the absence of intra cellular cryoprotection agents (CPAs) and that a high survival rate of RBCs in freeze dried state can be achieved.**
By employing a new freezing apparatus, "Multi-Temperature-Gradient" (MTG)[vi], we aim to overcome the above-mentioned problems and to enable the cryopreservation of large-volume samples. This freezing technology is based on **directional freezing**, in which the blood cell sample is transported in a customized container through a linear temperature gradient, which ensures precise control of the cooling rate and ice crystal front propagation. Thus, **by controlling the velocity and morphology of the crystallization, mechanical damage caused during freezing of the blood cells is significantly reduced.** This method may also incorporate controlled seeding into the freezing process. When any liquid is cooled below its freezing point, it remains a liquid, in an unstable super-cooled state, until freezing starts at randomly distributed nucleation sites and spreads throughout the entire volume of the liquid. Employing the multigradient directional cooling and warming technology, the crystallization of the blood cells is controlled. Therefore, upon thawing the cells can be used, since complete viability and integrity is maintained. A significant advantage to the multigradient freezing method is the virtual elimination of CPA use. Consequently, freeze drying (lyophilization) of the sample without the risk of CPA toxicity is possible through the process of sublimation.

Specific Aims of this research

SPECIFIC AIM #1: BUILD NOVEL ANIMAL MODEL FOR TESTING IN VIVO RED BLOOD CELL (RBC) FUNCTIONALITY
We will be using a unique animal model during the course of our study to test human RBC functionality. The model we propose employs the use of nude (athymic) rats. We intend to do preliminary work at the onset of our study with the aim to establish an effective animal model for the *in vivo* stages of our research. In order to ensure adequate time for establishing a workable animal model, we intend to initiate this aim at the beginning of the research in Year 1.

SPECIFIC AIM #2: OPTIMIZE FREEZE THAWING AND FREEZE DRYING PARAMETERS FOR RBCS.
This comprises an *In vitro* study to determine how to freeze thaw and freeze dry RBCs with substantially less cell damage than is the case with conventional freezing methods and without the use of CPAs. We will:

- Test a number of cooling rates using the MTG technology to arrive at the optimal cooling rate for freeze thawing and freeze drying
- Optimize freezing solutions which, combined with the optimal cooling rate, will provide desired cell survival rates for freeze thawing and freeze drying
- Optimize drying conditions such as drying time and temperature

SPECIFIC AIM #3: DETERMINATION OF FUNCTIONALITY IN VITRO OF RBCS BASED ON MEASUREMENT OF THE METABOLISM AND OSMOTIC FRAGILITY OF THE CELLS FOLLOWING FREEZE-THAWING AND FREEZE DRYING
This is an *in vitro* study to evaluate the functionality of RBCs following freezing and thawing and freeze-drying based metabolic assays to determine cell robustness.

SPECIFIC AIM #4: TESTING IN VIVO FUNCTIONALITY OF FREEZE THAWED AND FREEZE DRIED RBCS IN ATHYMIC RATS
An *in vivo* study to evaluate the functionality of reconstituted human RBCs based on blood oxygen saturation following freezing and drying. Testing will be done on athymic rats using the model established in SA #1.

SPECIFIC AIM #5: UP-SCALING OF THE MTG FREEZING DEVICE AND THAWING TECHNOLOGY FOR USE WITH VOLUMES OF 200 ML TO 600 ML
The current MTG device has a maximum volume of 8.5ml. Here we aim to scale-up to a device suitabl for clinical use. This aim focuses on engineering and biological solutions to overcome the following problems

- Scale up of the freezing device
- Move from freezing in glass tubes to bags
- Adjust for heat transfer in two dimensions (upper and lower) as opposed to only one with the tubes
- Adjust for mechanical problems associated with movement of the bags through the cooling blocks

SPECIFIC AIM #6: OPTIMIZE FREEZE-THAWING AND FREEZE DRYING PARAMETERS EMPLOYING THE SCALED UP FREEZING DEVICE
We need to ensure that we achieve the same level of cell integrity, cooling rate and interface velocity with large volume bags and scaled-up freezing device as we achieved with small tubes and volume samples.

SPECIFIC AIM #7: DETERMINATION OF FUNCTIONALITY IN VITRO OF RBCS BASED ON CELL METABOLISM AND OSMOTIC ROBUSTNESS OF THE CELLS FOLLOWING FREEZE-THAWING EMPLOYING THE SCALED UP FREEZING DEVICE
Here we will test to ensure that we achieve equivalent cell metabolism and osmotic robustness employing the large volume bags and scaled-up freezing device as was the case previously.

SPECIFIC AIM #8: FULL SCALE UP OF THE MTG FREEZE DRYING TECHNOLOGY TO HIGH TOTAL VOLUMES OF (E.G. 600 ML) RBCS AND SOLUTION
Here we will conduct a scale-up of the freeze drying technology to manage volumes suitable for clinical use. Challenges will include achieving greater vacuum levels and lower temperatures in the condenser.

SPECIFIC AIM #9: TESTING IN VIVO FUNCTIONALITY OF FREEZE THAWED AND FREEZE DRIED RBCS FROM UP-SCALED DEVICE IN ATHYMIC RATS
Here we will repeat the *in vivo* study from SA#4 to ensure the functionality of reconstituted human RBCs following freezing and drying in the up-scaled devices. We will use the athymic rat model developed in SA#1.

B Background and Significance

1 Background

1.1 The need for CPA-free frozen and freeze-dried red blood cells

According to the National Blood Data Resource Center (NBDRC), US institutions collected more than 15 million units of RBC products (whole blood and packed red cells) in 2001.[vii] Although blood supplies may be adequate overall, seasonal and geographical shortages continue to plague the blood banking system. This is mainly due to three factors: the relatively short shelf life of liquid-stored blood (42 days), wide seasonal swings in blood collection capability and unpredictable short term transfusion patterns.

RBC units are usually stored in the anticoagulant-preservative CPD-A2 for up to 42 days at refrigerator temperatures, after which they must be discarded because RBC recovery falls below acceptable levels (<70%). As a result approximately 1 million units of RBCs are discarded each year in the USA.[viii]

Currently, approximately 100,000 units (0.75%) of donated blood are frozen[ix], enabling it to be stored for up to ten years. While this is useful for maintaining stocks of rare blood types and (rarely) as reserve stocks for emergency shortages in fresh supplies, it has significant drawbacks. Chief among these is the necessity of using intracellular cryopreservation agents (CPAs), such as glycerol which must be washed out of the blood on thawing, before it can be used safely. This washing process typically takes about 30 minutes, rendering frozen blood supplies an impractical solution for emergency use.

Both r frigerated and frozen RBC supplies are awkward and expensive to store, requiring storage within a precise temperature range and the use of large, costly and continuously monitored refrigerators and freezers. According to a study by th American Association of Blood Banks (AABB), a unit of donor blood costs the blood banks from $111 to $145 to collect, test and process.[x] The relatively short shelf life of refrigerated blood and the costs and inconvenience of both liquid, but especially frozen storage, also combine to limit the

frequency of autologous blood donation. Limitations of current blood storage in both the liquid and frozen state include: the lack of easy portability, relatively short shelf life for liquid stored blood (42 days) and the need for post thaw washing to remove CPAs in frozen blood. Two of these limitations affect th speed and flexibility to meet immediate demand, thereby l ading to the possibility of inadequate supply in emergency situations.

Cumulatively, these factors highlight the need for two key advances in RBC technology:

I. A freezing process that is not reliant on CPAs, such that frozen RBCs can be available for use immediately after thawing, thereby facilitating greater use of frozen RBCs and reducing the overall outdating rate.
II. The ability to store RBCs in a concentrated, convenient form that would allow long term storage at low cost and immediate availability of RBCs to emergency medical teams in the field as well as in hospitals.

The research project described in this application aims to meet both these needs by (i) developing a solution for freeze thawing (FT) without the use of CPAs and (ii) providing a solution for freeze-dried (FD) RBCs that can be reconstituted by the simple addition of water.

1.2 State of the Art / Existing Knowledge

1.2.1 Freeze-thawing

Current freezing methods for cryopreserving blood units require the addition of intracellular cryopreservation agents (CPAs), usually glycerol. According to the Technical Manual for the American Association of Blood Banks, there are three basic methods of red blood cell freezing: High Glycerol, Agglomeration and Low Glycerol. A comparison of these three methods across a number of attributes is provided in Table 1 below.

Attribute	High Glycerol method	Agglomeration method	Low Glycerol method
Final glycerol concentration (w/v)	Approx.40%	Approx.40%	Approx.20%
Freezing rate	Slow	Slow	Rapid
Storage temperature (max)	-65C	-65C	-120C
Deglycerolizing time (minutes)	20 – 40	35	30
Special deglycerolizing equipment required	Yes	No	No

Table 1: Comparison of three methods of RBC freezing

After the blood unit is thawed, the toxic CPAs must be washed out using cumbersome, non-portable, expensive machines. The entire thawing and washing process takes approximately 30 minutes and adds $100-$125 to the basic cost of the blood unit[x]. The three methods described in Table 1 each use different wash protocols, however, the principle is the same. Hypertonic solution is used first for equilibration of the cells followed by washing with progressively less hypertonic solutions. A number of commercially available instruments for batch or continuous flow washing can be used for these procedures. "The thawed unit is first diluted with a quantity of 12% sodium chloride solution appropriate for the size of the unit and is allowed to equilibrate for approximately 5 minutes. This initial dilution is followed by washing with hypertonic sodium chloride until deglycerolization is complete. The cells are finally suspended in an isotonic solution of 0.9% NaCl with 0.2% dextrose[xi].

This time-consuming washing procedure renders the use of frozen RBC virtually impossible in most mergency cases. Nevertheless, frozen-thawed RBCs have been in clinical use throughout the world for about 30 years, with approximately 100,000 units frozen annually in the US alone[ix]. During this period of time, there has been little change in either the fundamental technology and or use of frozen-thawed RBCs. Recently, there has been an important advance (the Haemonetics ACP 215 system) which permits extending the post-thaw shelf life from 24 hours to 15 days[xii]. It is not clear as yet whether this improvement will affect the quantity or the manner in which frozen-thawed RBCs are used.

1.2.2 Freeze-drying

Over the years, a number of groups have worked on freeze-drying of RBCs, with only very limited success. Volker Rindler and associates at Aachen University of Technology in Germany are one such group whose research does not employ new freezing technology. They prepared and solidified the RBCs according to a cryopreservation protocol which was adapted to the specific conditions required for freeze-drying, and used a

n w low-temperature freeze-drying device. They claim to show a significant dependence of recovery rate on shelf temperature during storage, whereby a shelf temperature reduction led to improvement of recovery.[xiv] Their research is at a very preliminary stage and does not appear to be leading towards the same research goals as our technology at least in part du to the fact that their work focuses on controlled shelf temperatures during drying and all experiments were conducted on very small volumes. They are not looking to improve the high cell damage statistics associated with the current freezing methods.
A group from the University of California, Davis has applied for a patent[xv] on a procedure whereby RBCs are treated to remove alcohol (cholesterol) from the cell and cell membrane, after which the cells have a low phase transition temperature range. They may then be loaded with trehalose which preserves biological properties during freeze-drying and rehydration. The chief disadvantage associated with this work is the necessity to alter the composition of the cells' membrane in order to achieve membrane permeability to trehalose. In our work we are not altering the cells in any fashion and only making use of additives such as dextran and EGCG. We hypothesize there will be minimized cell damage during freezing based upon the innovative freezing technology.
Another area of research related to successful freeze thawing and freeze drying of cells is the work being done in introduction of sugars such as trehalose into cells to protect them from lethal injury[xvi xvii]. These works involve manipulation of the cells to affect cell membrane permeability thereby enabling the intracellular loading of cryoprotectants.

1.3 IMT's Solution

In this project, we propose to demonstrate that:

I. RBCs can be frozen and thawed without the use of CPAs;
II. RBCs can also be freeze-dried and reconstituted to a usable state without either CPAs or need to interfere with cell membrane structure.

IMT's innovative freezing technology focuses on a novel approach to the freezing process in which cells (or tissues and organs) are moved through a directional multigradient freezing apparatus that transports the cells in a manner that avoids or minimizes cell damage during the freezing process. This new technology, called "Multi-Thermal Gradient (MTG)" – a patented proprietary technology of IMT - applies a very precise and uniform cooling rate e.g. in the range of 0.1°C /min across the sample[vi]. In addition, the apparatus may control ice crystal propagation through the sample by initial seeding and then by regulating the velocity of movement of the material to be frozen through the thermal gradient. The MTG apparatus works differently from the usual stationary freezing methods where freezing starts at randomly distributed nucleation sites and spreads throughout the entire volume of the liquid. In these conventional freezing methods, ice crystals propagate with an uncontrolled velocity and morphology, and may disrupt and kill the cells in the samples. The damage is mechanical in nature, caused by the decreased size of the unfrozen fraction (U) [1]. It has been shown that in the absence of cryoprotectants RBCs will be damaged at temperatures of -10°C while only high glycerol concentration will protect them from a decreasing (U). The MTG, by controlling the ice crystal propagation enables freezing to take place with a significant reduction in cell damage.
Recently, we showed that interface velocity and thermal gradient in a modified directional solidification system has been effective on the survival of spermatozoa and leukocytes[xviii xix]. A high correlation between ice morphology and survival of human lymphocytes frozen by directional solidification was demonstrated by Backman[iv]. We now hypothesize that this new freezing method will minimize the damage that occurs during freezing from crystallization and that this will allow higher survival rates after freezing and thawing[21].

With the ability to freeze RBCs successfully without the use of intracellular CPAs, we hope to be able to successfully freeze dry the frozen blood thereby achieving a state in which storage, shipment, inventory management and safety are optimized. In addition, it is anticipated that the process will allow sufficient cost savings to ultimately mean lower costs to hospitals and patients.

2 Significance

2.1 Imp rtance of the research

Both of the key streams of this project have th potential to radically change the existing model for storage, supply and availability of RBCs, in turn providing a number of important benefits to the Health industry and the general population.

The ability to freeze thaw RBCs without CPAs that we aim to achieve, will enable medical institutions to rely to a much greater extent than now on frozen RBCs, as thawing for use will take just a few minutes. This will relieve pressure on collection agencies as greater stocks may be built up and retained in frozen state, significantly reduce wastage and hav a major impact on patient care as even rare blood types will be available at short notice for emergency treatment.
Lyophilization of RBCs for simple reconstitution will provide all the aforementioned advantages, but also achieve a total change in paradigm in the way a blood bank system operates. This includes:

- Reduced (even to null) refrigeration equipment and costs required for blood storage
- Indefinite shelf life
- Significant reduction in required storage space (the lyophilized samples are 3-5% the weight of their liquid form and significantly less in volume as well)
- Can be easily stored in emergency vehicles and quickly reconstituted, enabling paramedics to commence transfusions to trauma victims in situ or en route to hospital, thus saving many lives
- Autologous RBC donations can be lyophilized, vacuum packed and then returned to the donor for use in time of need, obviating the need for time-consuming testing and matching of replacement blood. This would be beneficial, for instance, to soldiers on the battlefield. Other candidates for carrying their own freeze dried RBCs are tourists and diplomats traveling and living in areas of the world where the blood supply is less reliable. Indeed, FD autologous blood could become the standard for all transfusions, and even permit individuals to keep their own blood stored at home (with proper identification and storage parameters).
- Hospitals and blood banks can maintain smaller, more compact supplies of FD blood since they will not have to contend with expiration dates. The stock determinants will be based on ongoing needs statistics with a calculated backup supply for emergencies or unusual needs.
- Ease of transportation of RBC units will mean better backup supplies in unexpected need situations such as local emergencies

2.2 Health Relevance

The success of this project in freeze thawed and freeze dried RBCs may have a broader significance, beyond those advantages in the field of RBC supply and banking already mentioned. We believe that once proven in RBCs, the same technology will be equally effective in the freeze drying of other blood components as well, including leucocytes, macrophages and stem cells and platelets. With regard to stem cells in particular, prolonging the shelf life and eliminating frozen storage will greatly simplify the logistics of stem cell operations and will likely increase the demand for this product. Furthermore, the investigators are aware of some confidential recent research and therapies in the field that will require the immediate availability of the cells at a location that is close to or at the hospital where a patient requires the procedure. In order to provide these therapies, laboratories would have to be set up and maintained at each hospital where such therapies are offered. Freeze dried cells would remove this need, making the therapies readily available anywhere and solving the time and logistics elements involved.
Lastly, the success of cost effective freeze thawed and freeze dried RBCs, could enable even greater effectiveness in donor screening for transmissible agents, as donated RBC units could be withheld from circulation for say 6 months, pending a follow-up health check of the donor.

C Preliminary Studies

1 Principal investigator's preliminary studies

Dr. Amir Arav, the principal investigator, is the founder of IMT Ltd and has been conducting research in the fields of cryobiology and thermodynamics for many years, now specializing in the cryopreservation of cells and animal reproduction. Dr. Arav developed the novel Multi-thermal Gradient (MTG) system described below, upon which this project is based. He also invented the VitMaster, the first vitrification device that enables very high cooling in liquid nitrogen slush, patented in 1998[xx], and a robotic system for time lapse microscopy within an incubator to be used for monitoring development during IVF procedures, patented in 2000[xxi].
Dr. Arav leads IMT's R&D in blood cell freezing, oocyte vitrification, cartilage freezing and transplantation, whole ovary freezing and transplantation and whole organ (liver) freezing. In 2001 he used the MTG system for whole organ freezing of a sheep's ovary followed by transplantation back to the same animal.

In the area of semen freezing Dr. Arav has been instrumental in achieving for the first time the freezing of large volume sexed bovine semen[xxii] using the MTG technology. With equine semen he has been able to show that 50% of the stallions which hav non-fr ezable semen with conventional freezing m thods can be frozen with the MTG system. In cell freezing Dr. Arav is currently working on cryopreserved blood cells and in collaboration with scientists in Italy has produced embryos by nuclear transfer of freeze dried cells which were shipped to Italy at room temperature.

Dr. Arav is currently working on further development and application of the multi-gradient freezing technology and freeze drying in:

- Freeze Drying of Umbilical Cord Blood: To date he has achieved 40% viability of stem cells following lyophilization and re-hydration
- Freeze drying of Leukocytes: 80% of the leukocytes that were frozen with the multigradient technology maintained viability after thawing
- Whole ovary freezing: Results showed the ability to cryopreserve intact sheep and cow ovaries, resume hormonal cyclicity and enable oocyte aspiration after re-implantation.
- The freeze thawing of osteochondral allografts: To date this work has resulted in the successful implantation of freeze thawed allografts in 17 sheep. 13 out of the 17 are normally ambulatory after periods ranging between 3 to 6 months.

2 Innovative Freezing Technology

As described in Sections A & B and part 1 above, this research project is based on the use of directional freezing using Dr Arav's novel MTG technology. The MTG device is normally constructed with between 2 to 4 temperature domains within 280 mm copper blocks (Fig 1). The sample is placed into a special container and moved at a constant velocity (V) through the predetermined temperature gradient(s) (G= ΔT/d where ΔT signifies temperature differences and d is the distance between temperatures) resulting in a constant cooling rate (B) within a single temperature gradient, which is calculated according to the equation B=G*V. At the inception of the procedure, the sample may undergo an inducing (seeding) of crystallization at the tip of the container between the temperatures T1 and T2. The container is then moved across G2 and G3 at a constant velocity.

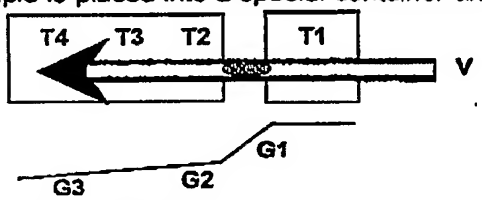


Figure 1: MTG freezing device

Freezing injuries

When a biological sample containing living cells is being frozen, there are two processes that cause damage: 1) intracellular freezing which causes cell death and 2) extra-cellular freezing that injures the cells due to mechanical pressure of the ice crystals on the cells (shearing). During freezing the cooling rate is thought to be the major factor that determines the survival of cells and tissue since the cooling rate determines the ratio of intracellular crystallization to extra cellular crystallization.[1] The rate of cooling also affects the morphology of the intercellular ice crystals[1]: Morphologies such as closely packed needles kill cells by external mechanical damage[2 3 4]. Thus, maximizing the survival rate of cells subjected to freezing and thawing requires careful control of the freezing process. The conventional slow-freezing method involves lowering the temperature of the chamber in a controlled stepwise manner. This method is based on using multidirectional (equiaxial) heat transfer to achieve a rate of temperature change in the sample that depends on the thermal conductivity, the geometrical shape of the container and of the biological material within it.[5] The thermal gradient within the sample is determined implicitly by the temperature of the chamber and the thermal conductivity of the materials of the sample, and is not directly controllable.

3 Program Results to date (*All materials unless mentioned otherwise were purchased from Sigma, USA.)

1. Mononuclear blood cells d rived from buffy c ats

One of our primary research goals is the development of freeze drying procedures for different cell types. This section describes our work to date in freeze drying of stem cells. Our initial work was with mononuclear cells, which contain mostly lymphocytes as our model. In the following experiments our goals were:

1. Finding the best cooling rate for freeze drying and freeze thawing.
2. Evaluating the influence of different concentrations of human serum albumin (HSA) on th viability of cells after freeze thawing and freeze drying.
3. Evaluating the influence of osmotic shock as a pre-freeze treatment on the viability of cells after freez thawing and freeze drying.

Materials and Methods

Most of the methods described in Section C will be used for experiments within Specific Aims #2, #3, #6 and #7.

Blood Separation

Buffy coats were received from Magen David Adom (MDA), Israel's National Blood Bank. The blood was separated on Ficoll-Paque gradient for 30 minutes at 1000g. Afterwards the mononuclear layer was drawn out and washed twice in Phosphate Buffered Saline (PBS) (calcium and magnesium free) for 10 minutes at 200g. To the pellet we then added our freezing solution at a volume of 2.5ml.

Freeze thawing and freeze drying

In our experiments 2.5ml of cell suspension were put in a 16mm diameter glass test tube (Manara, Israel) and frozen using the MTG freezing apparatus (IMT, Israel). All samples were rotated at 56 revolutions per minute (RPM) during freezing. After freezing, samples were plunged into liquid nitrogen and later were either thawed at 37°C in a water bath or put in a commercial lyophilizer (Labconco, USA) with a shelf temperature of -35°C and a collector temperature of -80°C. After drying, the cells were rehydrated with ultra pure water at 37°C.

Osmotic shock

Buffy coats that were received from MDA were either separated as above but not combined with a freezing solution (only PBS) or left untouched. To these two groups we induced two protocols of osmotic shock: *Treatment A*: The cells were exposed for 15 seconds to 0.5M trehalose in distilled water at a cell suspension to water ratio of 1:1.5 and then at the same ratio we added 1m trehalose in distilled water for 15 seconds. To end the shock we added 9%NaCl to reach a final concentration of 0.9% NaCl. *Treatment B:* to the two cell groups we added distilled water at volume ratio of 1:3 (cells to water) for 30 seconds. The shock was stopped by adding 9%NaCl in an amount to reach a final concentration of 0.9% NaCl. To these suspensions we added 0.5M trehalose.

After both treatments 2.5ml of the cell suspensions were put in glass test tubes and underwent freeze thawing and freeze drying as described above.

Viability assessments were done by evaluating the membrane integrity of the cells using SYBR14/Propidium Iodide (PI) (Molecular Probes, USA) live/dead fluorescent staining. Both stains are nucleic acid stains; SYBR14 is a membrane permeable molecule and PI can enter the cell only if the membrane is damaged.

DNA integrity assessment of the cells was performed using the single cell gel electrophoresis assay commonly known as the comet assay (Trevigen, USA). Cells were diluted to a concentration of 105cells/ml in PBS. The cells were combined with molten LM agarose at a ratio of: 1:10(v/v), then 75µl were placed on comet slides. The slides were kept at 4°C in the dark for 10 minutes. Slides were then immersed in a pre cooled (4°C) lysis solution for 1 hour at 4°C. Afterward, the slides were immersed in a freshly prepared alkali solution, which consisted of 0.6g NaOH pellets, 250µl of 250mM EDTA, pH 10.0 and 49.75ml deionized water for 60 minutes in the dark at room temperature. Finally, the slides were washed in a TBE buffer (Tris base, Boric acid and EDTA) for 5 minutes and then the slides were submerged in TBE buffer in a horizontal electrophoresis apparatus. We applied 1 volt per centimeter for 10 minutes. After the samples were dried they were stained with SYBR green. Scoring was done using a fluorescent microscope (Zeiss, Germany) connected to a digital camera (Sony, Japan) and analyzed using the Image J free software (NIH, USA).

MTG cryo-microscopy for detection of ice propagation was performed by placing a microscope connected to a CCD-camera above the MTG apparatus. The MTG apparatus was operated at different velocities and the ice propagation was captured.

Statistics

Means were calculated and differences between treatments examined by t tests using the JMP General Linear Model procedure[xxiii]. Significance was $P < 0.05$ unless otherwise stated.

Results

Our initial experiments were an valuation of the membrane integrity of cells after freeze thawing and freeze drying at different cooling rates. Our freezing solution in these experiments was composed of 0.1 M trehalose, 50% fetal calf serum (FCS) in PBS (calcium and magnesium free).

These results correspond to the classical inverted "U" shape where the intermediate cooling rate gave the best results.

Procedure \ CR	0.5°C/min	5°C/min	50°C/min
Freeze-thawing	52.8±1.9(a)	69.1±3.2 (b)	63.5±3.3% (c)
Freeze-drying	29.3±2.8 (d)	29.3±2.3 (d)	13.8±6.0% (e)

Table 2:

We also examined the influence of different interface velocities. Samples were frozen at the chosen cooling rate of 5.1°C/min but with four different interface velocities. After thawing there was no significant difference in membrane integrities:

Procedure \ Velocity	0.1mm/sec	0.2mm/sec	0.48mm/sec	1mm/sec
Freeze-thawing	69.3±1.2	66.9±1.7	64.1±2.9	69.6±3.0
Freeze-drying	7.8±1.1 (a)	21.1±1.1 (b)	12.0±1.0(c)	7.2±1.0%(a)

Table 3

Table 3 shows that after freeze drying the best ice interface velocity occurred at 0.2 mm/sec, which was our chosen velocity for all later experiments.

DNA integrity was compared using the comet assay described above. We examined cells after thawing and lyophilization; untreated cells (as a positive control) and cells that were treated with H_2O_2 (as a negative control). Freezing was done at a cooling rate of 5.1°C/min and interface velocity of 0.2mm/sec. Results were:

Untreated cells	Frozen-thawed cells	Frozen-dried cells	H_2O_2-treated cells
92.9±2.7(a)	71.9±2.7(b)	50.4±4.8(c)	5.6±1.4(d)

Table 4

We can see that after freeze drying although membrane integrity is ~30% the DNA integrity is ~50%.

The beneficial effect of proteins on lyophilized biological materials is known. Therefore, we wanted to examine the effect of adding human serum albumin (HSA) at different concentrations:

After lyophilization with 5% HSA there was extensive cell damage and the cell suspension became viscous. These results show that the percentage of cells with an intact membrane increased from ~70% to ~80% after freeze thawing. After freeze drying the increase in membrane integrity was small; from 30% to 35%.

Procedure \ % HSA	5%(w/v)	12.5%(w/v)	25%(w/v)
Freeze-thawing	69.4±2.1 (a)	76.8±1.6 (b)	61.2±1.4% (c)
Freeze-drying	29.8±1.2 (d)	31.5±1.1 (d)	26.9±1.4% (e)

Table 5.

Image 1 shows the ice crystal morphology at different ice interface velocities. We can see that when we increase the velocity the ice crystals become smaller and sharper with secondary branches. (The little circl s are leukocytes).

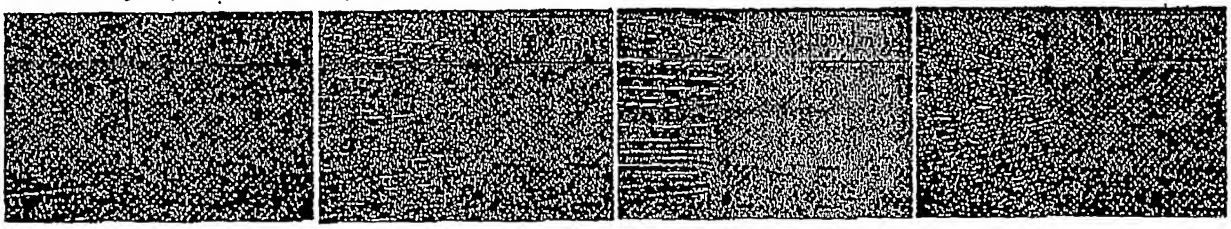


Image 1: Ice crystal morphologies at different ice interface velocities

Leucocytes were either separated on a Ficoll-Paque gradient or were left un-touched. They also underwent two different osmotic shocks (referred to as treatment A and B).

Procedure	MNC (Separated)		Leukocytes (Un-separated)	
	Treatment A	Treatment B	Treatment A	Treatment B
Freeze thawing	69.17±1.7%(bc)	58.98±1.9%(c)	89.09 ± 1.1%(a)	75.04± 1.9%(b)
Freeze drying	23.49±5.7%(f)	26.94±2.2%(ef)	34.86 ± 1.8%(e)	45.07 ± 1.9%(d)

Table 6

In Table 6 after inducing two different osmotic shocks on separated MNC's or un-separated leukocytes we can see that after freeze thawing the best result was obtained with leukocytes (Un-separated) after treatment A. After freeze drying best results were obtained with the leukocytes after treatment B.

To summarize: After osmotic shock treatments, freeze-thawed leukocytes showed ~75%-90% membrane integrity. The freeze-thawed MNCs showed ~60%-70% integrity. After freeze drying leukocytes showed ~35%-50% membrane integrity, depending on treatment. The results with MNCs were even lower and not significantly different between treatments.

Conclusions

The addition of HSA to the freezing solution showed an increase of about 5 to 10% in survival. After freez thawing we achieved 80% survival without cryoprotectants. In addition, according to comet assay results after freeze drying about 50% of the cells maintain their DNA structure intact.
Furthermore, we saw that un-separated leukocytes that underwent osmotic shock treatment display membrane integrity of approximately 50% after freeze drying. Because best results in both treatment cases were obtained by the group that was not separated on a Ficoll-Paque gradient, this suggests that there might be damage that occurs during the separation process. Our results, showing that we can freeze thaw leukocytes with ~80% survival without cryoprotectants and that we can dry the cells to about 5% water content and obtain 50% survival, are very encouraging.

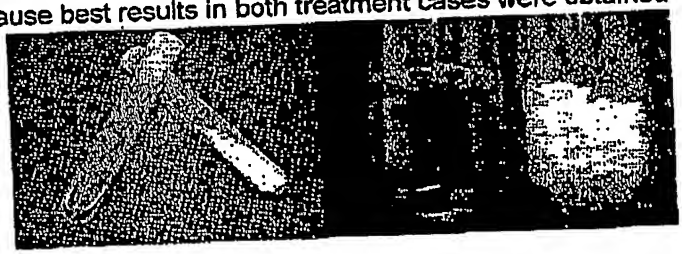


Image 2: Dry and rehydrated cells

2. Mononuclear blood cells derived from umbilical cord blood

After the experiments with cells obtained from buffy coats we began work directly with mononuclear cells from umbilical cord blood in order to evaluate optimal cooling rates and other parameters. In the following experiments our goals were:

1. Finding the best cooling rate for freeze thawing and freeze drying.
2. Evaluating the metabolism rate of cells after freeze thawing and freeze drying.
3. Evaluating the influence of adding epigallocatechin gallate (EGCG) to the freezing solution on the viability of cells after freeze thawing and freeze drying.

Materials and Methods

In the following experiments umbilical cord blood (UCB) was obtained from Sheba Medical Center. The blood was separated on a Ficoll-Paque gradient as described above. In these experiments we compared different freezing solutions containing:

1) 0.1M Trehalose, 12.5% (w/v) HSA in PBS (calcium and magnesium free).
2) 0.1M Trehalose, 12.5% (w/v) HSA and 51.5µM epigallocatechin gallate (EGCG) in PBS (calcium and magnesium free).
3) 12.5%(w/v) HSA and 51.5µM EGCG in PBS (calcium and magnesium free).
4) 51.5µM EGCG with 0.1M trehalose PBS (calcium and magnesium free).
5) 30% (w/v) Dextran with 51.5µM EGCG in PBS (calcium and magnesium free).

Samples were frozen, thawed, dried and rehydrated as described above. Viability was assessed using the live/dead fluorescent stains SYBR14/PI (Molecular Probes, USA) for membrane integrity. Cell metabolism was assessed using the cell proliferation kit (Biological Industries, Israel). This is a colorimetric assay which is based on the activity of mitochondrial enzymes and uses the tetrazolium salt XTT. Briefly, cells are cultivated in a flat 96-well plate. To each well we added 100µl growth medium consisting of: RPMI 1640 supplemented

with 10 fetal calf serum and 1% 200mM L-glutamine, 100units/ml penicillin and 100µg/ml streptomycin. In each well we had 2.5-5*106cells/ml. After incubation for 3 hours samples were read in an ELISA reader (Bio-Tek Instruments, USA) at a wave length of 450-500nm. Cell counts were performed using the automatic cell counter P ntra 60 (ABX Diagnostics, France).

Results:

In the first experiment, using MNCs suspended in a freezing solution composed of 0.1M trehalose and 12.5% (w/v) HSA in PBS (calcium and magnesium free), we compared different cooling rates:

C/R / Procedure	0.5°C/min	5.1°C/min	51°C/min
Freeze thawing	43.16±3.5 (a)	60.63±1.5 (b)	54.54±1.4 (c)
Freeze drying	16.79±1.4 (d)	25.21±2.7 (e)	7.5±0.9 (f)

Table 7

As expected, the best results were obtained at a cooling rate of 5.1°C/min for both freeze thawing and freeze drying. We also compared the cell metabolism rates after freeze thawing and freeze drying at the above cooling rates.

C/R / Procedure	0.5°C/min	5.1°C/min	51°C/min
Freeze thawing	28.32±4.8	33.83±7.6	23.63±2.9
Freeze drying	40.55±5.4	31.48±5.2	24.11±5.01

Table 8

This shows that there is no linear correspondence with the membrane integrity rates. At all cooling rates the percentage of cells that are metabolizing after freeze thawing is close to the percentage of cells that are metabolizing after freeze drying, indicating that the same percentage of cells maintain their capability to metabolize.

In the next set of experiments we compared different freezing solutions (see 1 – 5 above). All experiments were done at a cooling rate of 5.1°C/min.

Solution / Procedure	HSA& Trehalose	HSA &Trehalose & EGCG	HSA & EGCG	Trehalose & EGCG	Dextran & EGCG
Freeze thawing	56.27±3.3(ab)	47.86±4.2(abc)	25.34±2.7 (cd)	72.11±4.6 (a)	64.68±7.1 (ab)
Freeze drying	7.63±2.7 (cd)	9.11±2.2 (d)	2.4±0.78 (d)	51.75±2 (b)	24.42±2.6 (cd)

Table 9

The most obvious finding was that the group that contained trehalose and EGCG gave the best results. Dramatic improvement of membrane integrity is shown after freeze drying. Without EGCG the maximum percentage of membrane integrity was 10%, whereas, with EGCG percentage membrane integrity after freeze drying was 50% and after freeze thawing was 73%.

In the next set of experiments we compared the cell numbers before and after freeze thawing and freez drying with three different solutions: HSA and Trehalose, Dextran and EGCG and Trehalose and EGCG.

Solution / Procedure	HSA& Trehalose	Dextran & EGCG	Trehalose & EGCG
Freeze thawing	99	98	95
Freeze drying	90	63	96

Table 10

With all three solutions, after freeze thawing more than 90% of the cells survived. After freeze drying with trehalose and EGCG most cells survive. However, after freeze drying with dextran and EGCG only 62% of the cells survived.

C nclusions

We can see that interface velocity of 0.2mm/sec and a cooling rate of 5.1°C/min resulted in the highest membrane integrity rates after both freeze thawing and after freeze drying. These results are very similar to those obtained from buffy coats, indicating that the behavior of cells from both origins is similar. Furthermore, XTT results confirm that ~30% are viabl after freeze thawing and freeze drying.

The survival of mononuclear cells, which are mainly lymphocytes, suggests that hematopoietic stem cells, which morphologically resemble lymphocytes, also survive. However, we still need to examine this further. When we evaluat d different solutions with or without EGCG, it was immediately obvious that EGCG with trehalose gave the best results. Achieving membrane integrity of 50% after freeze drying without any intracellular cryoprotectant agents is very promising. We still need to evaluate the functionality and the ability of the hematopoietic stem cells to differentiate into different blood cells.

3. Red Blood Cells

The following experiments are part of an ongoing preliminary study being conducted prior to the full fledged study for developing procedures and technology for large scale freeze thawing and freeze drying of red blood cells. We have examined different cooling rates and different freezing solutions at different volumes (1, 2.5, 9, 50 and 200ml). For our experiment on freezing 200ml of cell suspension we have especially developed a prototype of an up scaled freezing apparatus.

Materials and Methods

Blood Separation

Whole blood units were received from the Israeli blood bank. The blood was centrifuged at 1500g for 10 minutes. After which, the supernatant was discarded and the erythrocytes were washed twice in PBS at 1500g for 10 minutes. Finally, to the packed erythrocytes (hematocrit approximately >80%) we added a freezing solution at a ratio of 1:1 and received a final hematocrit of 30-40%.

Freezing Procedure

The cell suspensions were frozen using the MTG freezing apparatus. Cooling rates evaluated in the first experiments were: 500, 1000, 2000 and 2500°C/min. The volume that was frozen was 1ml and freezing solutions that we examined were:

1) Saline
2) Autologous plasma
3) 30%(w/v) Dextran
4) 30% (w/v) Dextran and 51.5µM epigallocatechin gallate (EGCG)
5) 12.5% (w/v) HSA and 0.1M trehalose
6) 12.5% (w/v) HSA and 0.1M trehalose and 51.5µM EGCG
7) 51.5µM EGCG
8) 51.5µM EGCG and 0.1M trehalose

Further experiments were conducted at volumes of 9, 50 and 200ml. Thawing was done at 37°C in a water bath. Rehydration of the frozen dried samples was done by adding ddH_2O at 37°C to the original volume.

Morphology evaluations of the erythrocytes were done by observation of a 5µl drop of red blood cells under a light microscope (Zeiss, Germany) and by differential staining with May-Gruenwald Giemsa stains. Cell suspensions were spread on a glass slide and the slides were left to air dry. Then, slides were fixed in absolute methanol for 2-3 minutes at room temperature. Following, slides were stained for 15 minutes in May-Gruenwald stain that was freshly diluted with an equal volume of distilled water, pH6.8. After 15 minutes the slides were stained for 10 minutes in Giemsa stain that was freshly diluted with distilled water (1:9 ratio), pH6.8. Then the slides were washed in running tap water and left for 3-4 minutes in distilled water. After which, we allowed the slides to dry and then observed them under a light microscope (Zeiss, Germany) using immersion oil.[xxiv]

Cell numbers and Hematocrit: The number of red blood cells and hematocrit were measured before and after each procedure, using the automatic cell counter Pentra 60 (ABX Diagnostics, France).

Scanning electron microscopy (SEM) was performed on frozen dried samples. The samples were gold plated before being placed in the SEM. The voltage of the electron scatter was 25kv. The electrons hit the sample and returned to a detector and a 3D picture was displayed on a CRT screen.

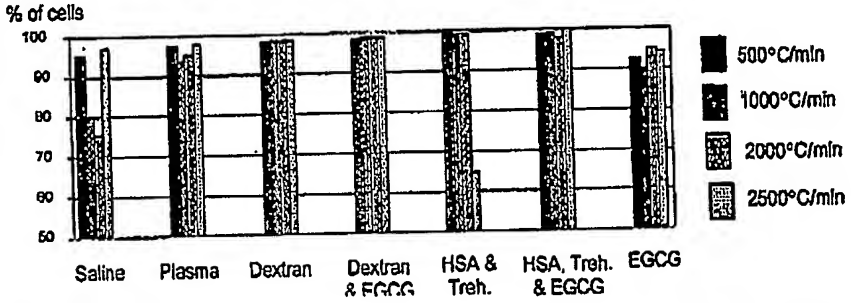
FTIR Microscopy: Bruker-Equinox 55 Fourier transform infrared (FTIR) spectrometer connected to a Bruker FTIR microscope A590 (Ettlingen, D-76275, G rmany) was used to observe membrane properties of fresh and frozen thawed erythrocytes. The FTIR microscope sends an infra red signal to the samples, this in turn causes the molecules to vibrat and th infra red light is returned at a different wave length. These vibrations are unique and th y are a result of the atom connections that were disturbed by the infra red light. The spectrum ranges in which w were interested were around 2870 cm-1 which is the C-H of hemoglobin and around 1650 cm-1 which is probably the protein secondary structure that depends on metal ions (1).

Results

RBC survival after freeze thawing at a v lume of 1ml

The survival of RBCs that was detected by the automatic Pentra 60 counter is shown in fig. 2. At all the tested cooling rates dextran or dextran with EGCG protected the RBCs better than other solutions.

Fig. 2: The survival rate of the erythrocytes after freeze thawing at different cooling rates and with different solutions, frozen at a volume of 1ml



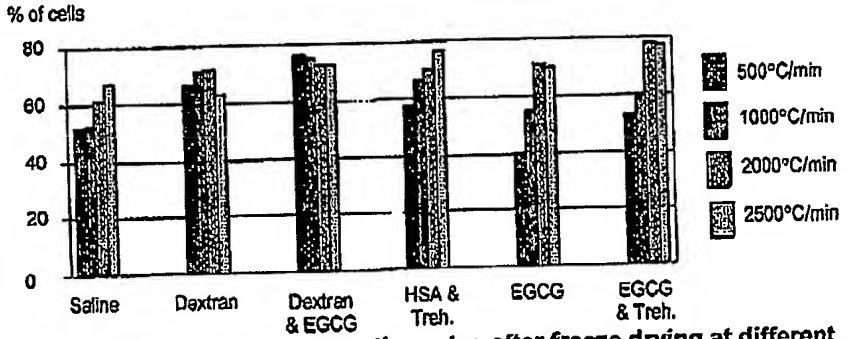


Fig. 3: The survival of the erythrocytes after freeze drying at different cooling rates and with different solutions, frozen at a volume of 1ml

RBC survival after freeze drying at a volume of 1ml

Figure 3 describes the effect of different solutions on the survival of the cells after freeze drying. We can see that the samples that contained dextran and EGCG protected the RBCs at all cooling rates, whereas with other solutions higher cooling rates gave better results (except for the solutions with dextran).

Survival of RBCs after freeze thawing and freeze drying at a volume of 2.5ml

Fig. 4 illustrates the percentage of erythrocytes that survive after freeze thawing and freeze drying with different solutions at a cooling rate of 1000°C/min and at a volume of 2.5ml. We can see that solutions with dextran gave the best results after freeze thawing and freeze drying. There is no significant difference between freezing at a volume of 1ml to freezing at a volume of 2.5ml.

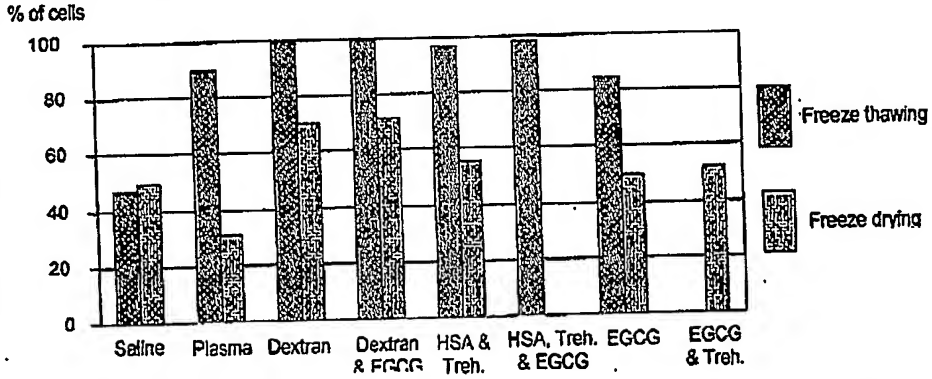


Fig.4: Survival of erythrocytes after freeze thawing and freeze drying with different solutions at a cooling rate of 1000°C/min and at a volume of 2.5ml.

RBC survival after freeze thawing at a volume f 9ml

Figure 5 shows, again, that solutions with dextran and EGCG protect RBCs at all cooling rates and that the survival of RBCs frozen with saline increas s with cooling rate. In addition, freezing at 9ml shows no significant differences than freezing at 1 or 2.5ml.

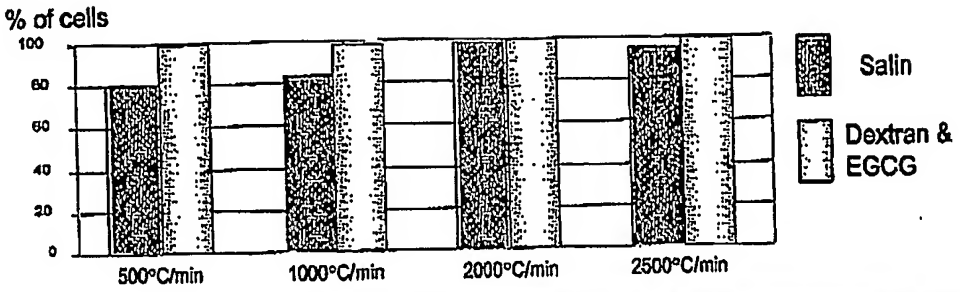
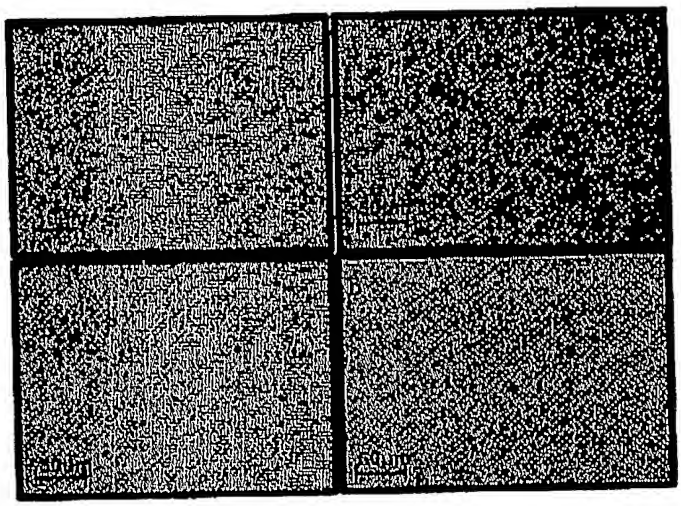


Fig.5: Th survival of the rythrocyte's number after freeze thawing at different cooling rates with two different solutions at a volume of 9ml.

Image 3: Frozen-Thawed Erythrocytes

These pictures were taken after freeze thawing of 9ml erythrocytes at 1000°C/min. Pictures A and B were frozen with a solution composed of Dextran and EGCG. Pictures C and D were frozen with saline.



The effect of storage time at -80°C on survival of frozen thawed RBCs

Figure 6 shows that after 3 days of storage the number of surviving erythrocytes frozen with saline decreases, whereas, erythrocytes frozen with dextran and EGCG maintain their cell numbers.

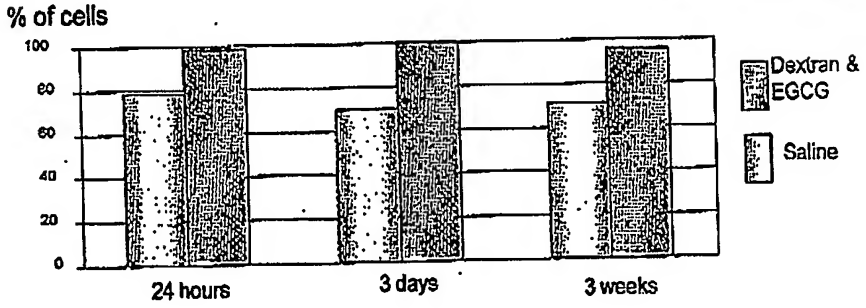


Fig.6: The survival rate of erythrocytes after being frozen at a volume of 9ml and stored at -80°C for up to 3 weeks.

The survival of RBCs after freezing and thawing at a volume of 50ml

When scaling up the volume to 50ml, a slower cooling rate of 330°C/min gave the best results. It is important to note that in this experiment the thermal gradient remained constant (G =5.5°C/mm) while only the interface velocity ranged from 0.5 to 3mm/sec, thus the cooling rate was changed respectively.

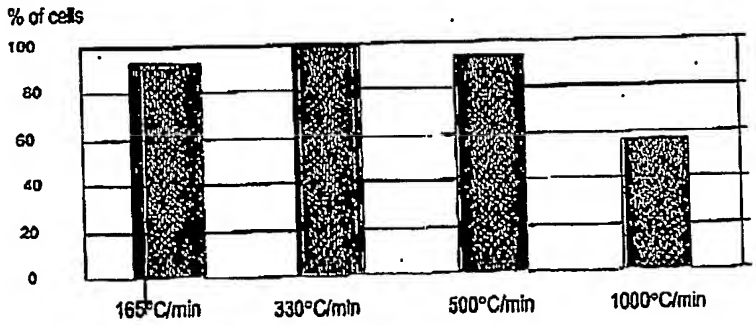


Fig. 7: The survival rate of the erythrocytes after freeze thawing at a volume of 50ml with a freezing solution containing Dextran and EGCG at different co ling rates.

RBCs survival after freeze drying at a v lume f 200ml in a freezing bag

In an experiment where we froze RBCs using a plastic bag and a new prototype MTG device, we achiev d almost 80% cell survival and hematocrit (Our initial cell number was $2.7x10^9$cells/ml and hematocrit level was 22.7%) as illustrated in figure 8.

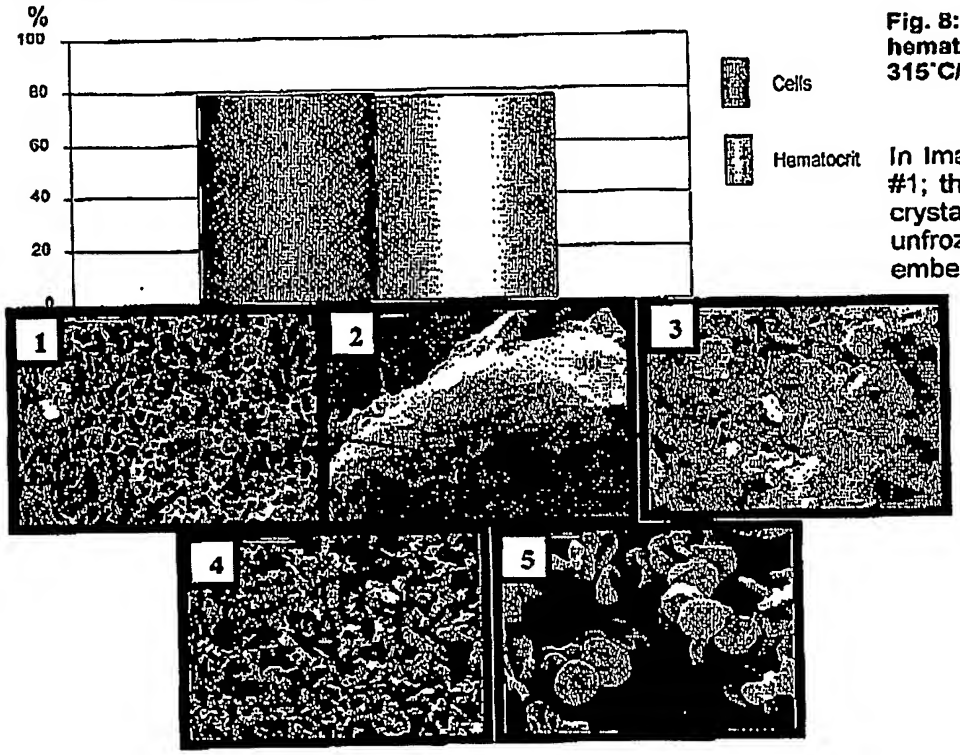
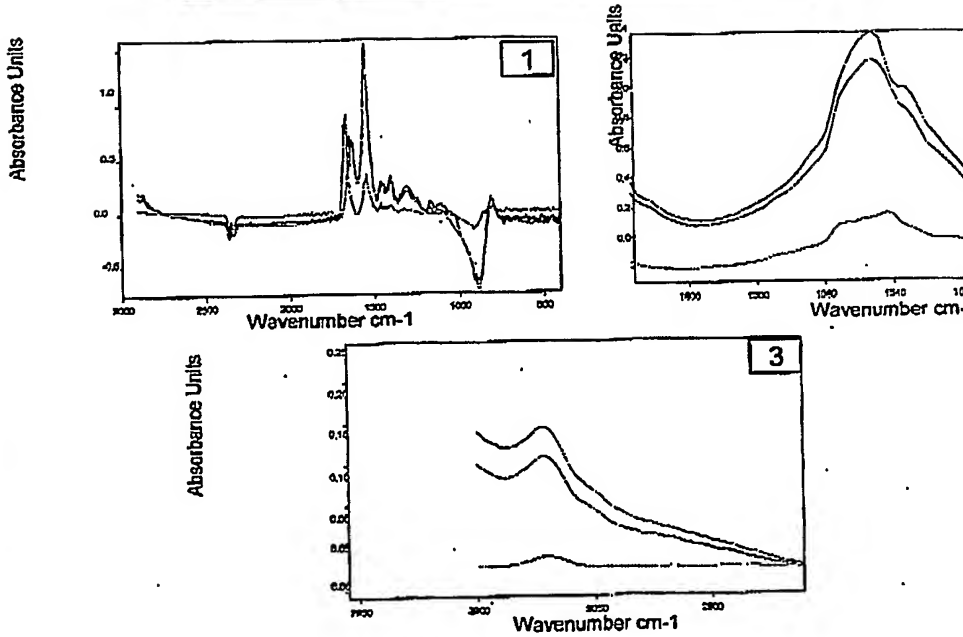


Fig. 8: Percentage of erythrocytes and hematocrit after freezing (200ml, CR 315°C/min, conventional freezing bag)

In Image 4, #2 is an enlargement of #1; this is the area between the ice crystals which is known as the unfrozen fraction (U). The RBCs are embedded in the dextran in this area (U). Whereas, in #4 and #5 there is no matrix to protect the cells but morphologically they seem normal.

Image 4: Membrane and protein structure of RBCs after freeze thawing

These are SEM pictures of frozen dried erythrocytes. Numbers 1 to 3 are erythrocytes frozen with 30% (w/v) Dextran and 51.55µM EGCG. Pictures 4 and 5 are erythrocytes frozen only with saline



Green – Fresh red blood cells
Red – Frozen thawed red blood cells with dextran and EGCG
Purple – Frozen thawed erythrocytes with saline

Fig.9: FTIR microscopy measurements of fresh and frozen thawed erythrocytes.

(A color copy is included in the appendix).

Erythrocytes were frozen with a solution composed of Dextran and EGCG or with saline. #1 shows the structure integrity. #2 shows the spectra 1500-1600cm-1 which indicates the protein secondary structure in connection to metal ions. #3 shows the peak at 2870cm-1 which indicates the C-H of hemoglobin.

Conclusions

The results presented in the section on Red blood cells indicate that in the absence of intracellular cryoprotectants fast cooling rates such as 1000 and 2000°C/min give better results for freezing and thawing and freeze drying in small volumes up to 9ml (figures 2-5). However, when we froze larger samples (this is very obvious when freezing 50ml samples) lower cooling rates of 330 and 500°C/min (fig. 7) gave better results. This corresponds with the knowledge that in larger masses there is slower heat transfer conductivity.
In order to enable freezing of a large volume (200ml) in a conventional freezing bag we had to develop a specially designed MTG machine. The cooling rate was 315°C/min, which gave best results when freezing 50ml of blood and resulted in 78% survival of the cells (fig.8). These preliminary results are very promising with regard to the up-scaling of the MTG machine and the possibility of freezing large volumes in the absence of intracellular cryoprotectants. Rapatz et al.[xxv] conducted experiments with red blood cells in the late 60s where they compared different cooling rates from 1 - 15,000°C/min with different freezing solutions (containing intracellular cryoprotectants or without). They found that with intracellular cryoprotectants the optimal cooling rates were between 5 - 150°C/min (with 10% glycerol or DMSO) and increased to 1600 - 3500°C/min without cryoprotectants. We can see that these results correspond to our results where with higher cooling rates we achieved better survival. However, Rapatz et al. did their experiments with very small volumes (in capillaries). When increasing the volume we saw better results at lower cooling rates. **We hypothesize that the directional freezing has an effect similar to that of the intracellular cryoprotectants in increasing the unfrozen fraction at a slow cooling rate, thus enabling high survival of RBCs at levels closer to those that would be used if we had glycerol in our solution.**

When freeze drying at different cooling rates with different solutions (fig.3), we can see that the samples containing dextran are not influenced by the changing cooling rates, whereas, with the other solutions the viability increased with the cooling rate. In figure 4 it is obvious that the best solutions for freeze drying are those that are with dextran. Rindler et al[xxvi] [xxvii]conducted freeze drying experiments where they compared whether the cooling rates that are optimal for freeze thawing are the same cooling rates that are optimal for freeze drying. They found that for freeze drying very fast cooling rates gave better results. In our experiments we do not see a difference between the best cooling rates for freeze drying or freeze thawing (figures 2 to 4). Rindler's group also examined the drying process, especially the optimal chamber temperature of the lyophilizer (they checked shelf temperatures between -5 and -65°C). They found that the optimal temperature is -35°C. Our samples were processed at a chamber temperature of -35°C; and one of the ways that we plan to try and minimize the damage that occurs during primary drying is through reducing the temperature to -100°C. This temperature is below the recrystallization temperature (which is -40°C) and below the glass transition temperature (which is ~-80°C). We believe that at such a low temperature we will prevent de-vitrification and re-crystallization damage. Furthermore, the number of cells that survive is lower after freeze drying than freeze thawing and the hematocrit level (data not shown) is also lower. This indicates that there is hemolysis. The hematocrit percentage after freeze thawing is higher than that of the fresh cells. In addition, the hematocrit level after freeze thawing is also higher than after freeze drying (data not shown). We assume this is caused by two factors: 1) that there is more hemolysis of cells during drying, and indeed their number decreases. 2) the mean cell volume (MCV) (data not shown) of the cells has increased after freeze thawing, thus resulting in more then 100% hematocrit compared to the hematocrit level before freezing.
In figure 6 we have evaluated the effect of storing the frozen samples at -80°C instead of in liquid nitrogen. At this point we have only evaluated this for up to 3 weeks and we can see that the cell numbers did not decrease for cells that were frozen with dextran and EGCG. The only change is the hematocrit which almost reached its original level after 3 weeks.

We can see that there is not a great difference in the number of cells after freeze thawing. However, after evaluating the morphology of the cells by differential staining and by observation in a light microscope it was clear that there are differences between the solutions and that the automatic cell counter Pentra 60 counts ghosts (cells with only their membrane and without hemoglobin). The solution that gave the best results according to microscopic evaluation after freeze thawing and freeze drying was composed of dextran and EGCG. This solution resulted in red blood cells that morphologically seemed much better than other solutions after both freeze thawing and freeze drying. Some of these photos are shown in pictures 3and 4.
Scanning electron microscopy that was done on erythrocytes that were frozen with either saline (as a negative control) or with dextran and EGCG and th n dried, revealed intact cells. The most apparent difference is that cells that were freeze dried with saline have no matrix. We can see many erythrocytes just scattered. Howev r,

when looking at samples with dextran and EGCG we see that the cells are embedded in the matrix. The dextran enwraps the cells and stabilizes them. In picture 5 no.1 we can see where the ice crystals that were sublimated used to be and the patterns confirm directional propagation of the ice crystals.

In comparison of C-H of hemoglobin and protein properties by FTIR (Fig.9) we can see that there is no difference in the pattern between frozen thawed cells (dextran and EGCG group) and fresh blood. However, the cells that were just frozen with saline have a smaller surface area beneath the peaks. That indicat s changes in the C-H of the hemoglobin and changes in the secondary structure of the protein. We assume that the differences between the samples are, in addition to the stabilizing dextran effect, due to the addition of EGCG and that it interacts with the membrane and might even change the lipid phase transition temperature of the cells. These assumptions will be investigated in the proposed study.

Our results indicate that high cooling rates give better results for freeze thawing and freeze drying. In addition, the solution composition is crucial to the survival of cells to as great an extent as the cooling rate. We have seen that the combination of EGCG (an antioxidant) and dextran resulted in higher survival rates and improved morphology.

D Research Design & Methods

SPECIFIC AIM #1: BUILD NOVEL ANIMAL MODEL FOR TESTING IN VIVO RED BLOOD CELL (RBC) FUNCTIONALITY

Rationale

Following the *in vitro* experiments described below (Specific Aims #2, #3, #7, #8), we plan to study the survival and functionality of our FT and FD red blood cells *in vivo* before proceeding to clinical trials. For this purpose we need to use nude (athymic) rats. Nude mice are also theoretically a viable study model, but their size makes repeated blood removal and transfusion not applicable. Since we are unaware of any previous use of athymic rats to study human RBCs, we need to develop and thoroughly test this animal model at the outset, in order to rely on it for Specific Aims #4 and #9. If we are successful in building a working animal model, then it may be applicable to other areas of research as well.

In building this animal model we will need to ensure that the reduced immune system in the nude rats does not react adversely to human blood transfusions. We will also need to practice the surgical procedures required to remove blood from and transfuse blood to the rats.

Methods

Ten athymic rats will be purchased and kept at our animal house. In order to determine the feasibility of our animal model for testing of human blood we will:

1. Canulate the central vein under anesthesia one week before the experiment
2. Remove blood samples from the rats (2ml)
3. Each sample will be centrifuged and the plasma will be returned to the rat
4. The rats will undergo autologous transfusion of RBCs

Following these initial trials, we will test the rats with transfusions of human RBCs:

1. 2-3ml transfusions of human RBCs will be made to a number of rats and their reactions will be monitored (inc. heart rate, pO_2, behavioral signs). Any signs of illness or distress will be considered negative reactions.
2. If there are no negative reactions to the human RBC transfusions, we will conduct a small scale experiment with the Samarium RBC kit (BioPAL Inc. USA) for RBC labeling in order to check the survival of human RBCs in rats. Human blood samples will be labeled with these isotopes and then transfused back into the nude rats (8 – 9 weeks old). Then blood samples will be drawn from the rats in order to evaluate the amount of the circulating RBCs. The assay will be performed by BioPAL Inc. (Worcester MA) using neutron activation technology. This technology is based on the principle that an incident neutron is captured by an atom forming a radioactive nucleus. The isotopic nucl us releases gamma-rays; the energy of th gamma-ray is discrete and distinct for each stable atom. Specialized high resolution detection equipment is used to measure the emitted gamma-rays. The number of emitted gamma-rays is directly proportional to the total mass of the parent isotope, and therefore is

proportional to the total concentration of the labeled research product (in our case human erythrocytes) contained in the sample.

Blood will be collected and transfused at the jugular vein using an indwelling intravenous catheter. Although this involves a difficult surgical procedure, it is necessary in order to perform repeated blood tests. The PI, a qualified veterinarian, will perform the surgery. The following procedures will be performed:

Catheter preparation: We will use common catheter materials including polyethylene, silicone rubber and polyurethane as well as a catheter guide, which provides rigidity to the catheter, facilitating introduction and placement in a blood vessel. The end of the catheter will be fitted with a standard connector that is compatible with the hub of standard syringe sizes.

Surgical procedure:
The animal will be anaesthetized using 10mg/kg xylazine and 90mg/kg ketamine. The anaesthetized rat will be laid on its back with the head toward the surgeon. The catheter will be advanced 3 – 4 cm to ensure proper seating of the catheter. The rat's hair will be shaved on the ventral neck from the midline and extending laterally 1cm past the indented jugular "groove", cranial to the angle of the jaw and caudal to the scapula. The skin will be swabbed with alcohol. Using aseptic technique, a 1.5-2mm incision will be made in the ventral neck to one side of the midline. The fat and the connective tissue will be cleared gently by blunt dissection.
A pair of mosquito hemostats or a needle driver will be passed under the vein and two pieces of suture (silk) will be placed around the vein. The anterior ligature will be tightly tied to completely occlude the vein and the posterior ligature will be placed loosely around the vein. The vein will then be placed under light tension by raising the posterior ligature vertically and the vein will be semi transected with ophthalmological scissors at a point between the two ligatures. A sterile heparinized saline-filled catheter (20 units heparin/ml) will be introduced into the vein and advanced towards the heart. The posterior ligature will then be tied snugly around the catheter and vein. Catheter position will be verified by attaching a sterile saline-filled syringe to the metal connector of the catheter. If blood withdrawal is satisfactory, it will be flushed back into the circulation and the catheter filled with heparinized saline (20 units heparin / ml) in order to provide a "heparin lock". A third ligature will be placed and between the first and second ligatures, where the catheter exits the jugular vein, the catheter will be tied to the vein. To exteriorize the catheter, the dorsal neck will be shaved and the incision site cleaned using alcohol. A 0.5 – 1.0 cm incision will be made and a straight pair of hemostats will be used to create a subcutaneous tunnel extending from the dorsal neck incision along the side of the neck to the entry point of the catheter.
The catheter will be grasped and a generous loop "stress loop" will be created in the area of the subcutaneous pocket near the catheter exit point in order to provide slack to minimize disruption of the catheter and to accommodate an animal's normal movements. The catheter will then be cut with 2.5 – 3.0 cm exteriorized and the end capped. The skin incisions will then be sutured and a sterile topical antibiotic applied. The neck will b wrapped with a light bandage to incorporate the catheter.

Is topic labeling of RBCs using the Samarium RBC kit (BioPAL Inc. USA). Human blood samples will be labeled with these isotopes and then transfused back into the nude rats (8-9 weeks old). Then, blood samples will be drawn out at periodic intervals over approximately 10 days from the rat in order to evaluate the amount of the circulating RBCs. The assay will be performed by BioPAL using neutron activation technology.

Evaluation of oxygen saturation level: A quantity of blood will be removed from the nude rats and centrifuged for 10 minutes at 1500g, to separate out the plasma. The plasma only will be re-transfused in order to cause a reduced level of blood oxygen saturation without reducing blood volume. We will then transfuse freeze thawed or freeze dried RBCs, at the same volume of packed red cells as that which was removed, and then measure the oxygen saturation level of the blood to determine if the level has risen.

Urine tests will be performed to detect blood in the urine which would indicate poor survival of the transfused RBCs.
Following conclusion of the experiments the rats will be sacrificed with injections of Pentobarbitone to the vein.

Alternative
We will begin our project with the work on this unique animal model in order to ensure its feasibility. If we do not receive the anticipated results with the nude rats then following Specific Aim #8 we would move directly to human subject testing (not within the scope of this application).

SPECIFIC AIM #2 – OPTIMIZE FREEZE-THAWING AND FREEZE DRYING PARAMETERS

Rationale

In our preliminary studies (described in section C above) we obtained good survival rates for RBCs following both freeze-thawing and freeze drying. The results were obtained on individual and very small volume samples. It is, therefore, necessary to prepare the ground for up-scaling of the freezing and lyophilization technologies for larger volumes.

The challenge being met in SA#2 is to optimize the experimental parameters (cooling rate and solutions) to the point where cell damage is minimal without the use of intra cellular CPAs. Based on the results obtained in our preliminary studies we have established a range of parameter values from which we will begin our testing in order to establish the optimal values.

Methods

Whole blood will be obtained from MDA. In order to obtain packed red cells we will perform the following process:

1. The blood will be placed in a centrifuge for 10 minutes at 1500g.
2. The plasma will be drawn out and PBS (Ca and Mg free) added.
3. The blood will be centrifuged again for 10 minutes at 1500. This washing step is done twice. At the end we will get packed red cells, at about 80-90% hematocrit.

The packed cells will be mixed with a freezing solution (see below) at an equal (v/v) ratio. Freezing will be p rformed in glass test tubes using the MTG freezing apparatus. Samples to be frozen and dried will be spun during the freezing process, in order to achieve a large surface area.

The following compositions of freezing solutions will be evaluated:

1. Dextran and epigallocatechin gallate (EGCG)
2. Dextran and vitamin C
3. Dextran, vitamin E and an emulsification ingredient

The interface velocities (V) will be: 0.5,1,1.5 and 3mm/sec. The thermal gradient (G) will be 5.5 and 10.5°C/mm resulting in the following calculated cooling rates (CR): 165, 320, 500, 630, 1000 and 2000°C/min. The volume of the cell suspensions will be 9 ml and 50ml at this stage.

The experimental parameters will be checked with the following tests:

1 MTG cryo-microscopy – evaluation of ice crystal morphology with the different thermal parameters and different freezing solutions

Cryo-microscopy for the detection of ice morphology will be performed by placing a microscope connected to a CCD camera above the MTG apparatus (see Figure 10). In the MTG device there is a gap between the freezing blocks where the microscope can be situated with the camera above it. This will be effective in evaluation of the freezing process of RBCs.

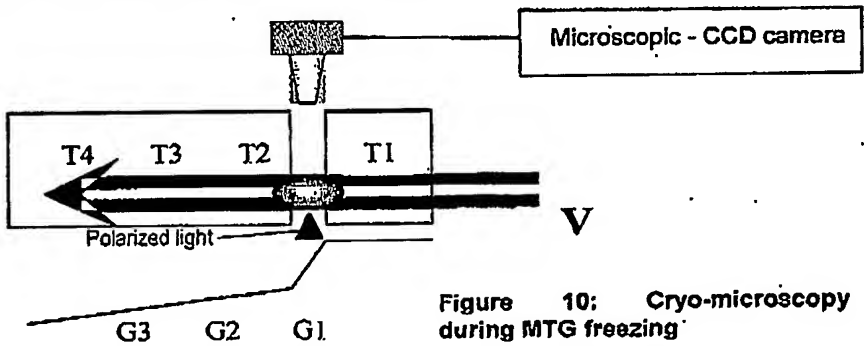


Figure 10: Cryo-microscopy during MTG freezing

For freeze dried cells, the sublimation process will be evaluated using cryo-microscopy. The specially designed MTG cryo-microscope will be employed: T1 is the temperature above the freezing point; T2 is the temperature below the freezing point. Between T1 and T2 the microscopic-CCD camera evaluates the freezing process and the morphology of the ice crystals (which depends on interface velocity). A special rectangular-glass-tube containing the sample is moved through T1, T2 and T3 at a constant velocity[8].

2 Complete blo d count (CBC)

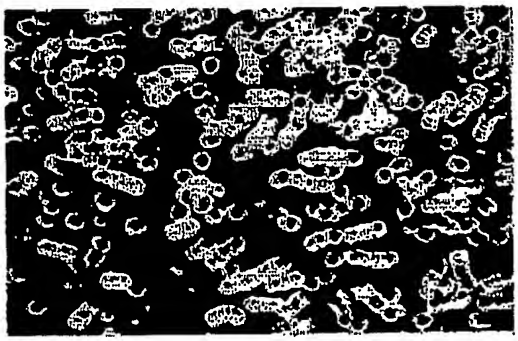
The CBC will be measured using an automated counter (Pentra 60, ABX, France) which will indicate a number of parameters such as the number of cells. Mean cell volume (MCV) and the hematocrit will be examined.

A limitation of CBC is that the procedure can show ghost cells that are membranes only – i. . it also counts dead cells. The presence of ghosts indicates that the cell membrane has been damaged and the Hb has leaked out of the cell, but th cell has not undergone hemolysis (the complete disintegration of the cell).
To compensate for ghost c ll inclusion in the CBC, and other possible shortcomings of any single procedure, we will perform a series of procedures for evaluation of cell morphology and viability as described in this section. The results when taken together should provide a reasonably accurate picture of cell survivability after FT and FD.

3 Differential stainings
May-Gruenwald Giemsa Stainings will be performed to evaluate cell morphology after freeze thawing and freeze drying.

4 Morphology assessment with light microscopy
Morphology assessment will be performed to determine cell appearance and the rate of hemolysis after each procedure. 5µl of cell suspension will be placed under a light microscope. In examining the cells (at a magnification of up to X400) we should see the cells as they are while the ghosts appear as much more transparent cells than normal erythrocytes.

5 Scanning electron microscopy (SEM) – for morphology evaluation after freezing and freeze drying
SEM will be performed using an electron microscope on both the frozen/thawed and frozen dried samples. The samples are gold plated before being placed in the SEM. The voltage of the electron scatter is 25kv. The electrons hit the sample and are returned to a detector and a 3D picture is displayed on a CRT screen. We expect to see that the RBCs are embedded in the matrix in a manner that is similar to that which is described as the arrangement of erythrocytes in the Rouleaux effect[xxviii] with normal morphology and discoid shape. (See example of the Rouleaux effect in Picture 6 below.)



We expect to see this effect or manner of cells organization during our directional freezing. As freezing commences we theorize that most of the cells will be pushed by the ice crystals into the unfrozen area (between the crystals) causing them to be aligned and to form compact orderly structures that seem like the Rouleaux effect. Because this occurs in the unfrozen area, there should be higher survival rates. With the optimal cooling rate, this unfrozen area should be greater, thereby allowing more cells to be included and thus survive.

Image 5: Example of red blood cell aggregation and Rouleaux formation

6 Membrane properties and dehydration measurements
Membrane properties such as C–H connection of hemoglobin and protein structure (approximately 1560 on th spectrum) will be evaluated. We will also weigh the samples before and after sublimation to validate that the weight loss experienced derives from loss of water.
The evaluation will be performed as follows: We will use a Bruker-Equinox 55 Fourier transform infrared (FTIR) spectrometer connected to a Bruker FTIR microscope A590 (Ettlingen,D-76275,Germany). A pellet of the cells will be placed between two sapphire windows.[xxix]. In order to know if the weight loss is actually water loss, we will use the FTIR microscope again. The nominal spectral resolution that will be used is 4 cm-1 and 66 scans, which are co-added per sample spectrum. The cells will be placed between two sapphire windows. Data is processed to obtain FTIR spectra in the vibrational frequency of bonded OH groups. This spectrum which is between 2800 and 3800 will be calculated using the OPUS software.

Expected results:
We expect values to match those of fresh RBCs. If we do not achieve these expected results then we will continue our testing with additional CR values that are above and below the range indicated in section C above. We will also make adjustments to the cooling solutions in order to achieve the desired results.

We will employ several somewhat overlapping procedures with the intent to obtain a complete picture of cell survival. The differential staining procedure provides additional information which can identify th ghost cells

not apparent in the CBC. Microscopic cell counting and SEM will provide more data in the freeze dried samples enabling a picture of the entire matrix in the dry state.

SPECIFIC AIM #3: DETERMINATION OF FUNCTIONALITY IN VITRO OF RBCS BASED ON CELL METABOLISM OF THE CELLS FOLLOWING FREEZE-THAWING AND FREEZE DRYING

Rationale
In SA #2 we will fine tune the freeze thaw process, testing the morphological and membrane integrity of the cells. In Specific Aim #3 we need to determine if these cells are functional *in vitro* and whether they retain their metabolic functioning.

Methods
We will perform metabolic assays of Adenosine triphosphate (ATP), 2,3-diphosphoglyceric acid (2,3-DPG) to show metabolic activity and membrane robustness.

I. **The metabolic assay of ATP** will be performed using the ATP Bioluminescence Assay Kit HS II (Roche)
II. **The metabolic assay of 2, 3-DPG** will be performed using the 2,3-Diphosphoglycerate UV-test for the determination of 2,3-DPG in blood research samples (Roche).

Interpretation of results
The metabolic assays will be performed after determination that the cells survive and appear viable. It will indicate if these cells can still accomplish their critical metabolic tasks.
In addition, we will test additions of additives to the solution which could include different antioxidants such as vitamin C or E. We may also try to insert trehalose into liposomes followed by fusion with the RBCs or hypo-osmotic shock of the RBC to increase survival after freeze drying, and retest with the optimal cooling rate as well as other cooling rates.

SPECIFIC AIM #4: TESTING IN VIVO FUNCTIONALITY OF FREEZE THAWED AND FREEZE DRIED RBCS IN ATHYMIC RATS

Rationale
Following the in vitro testing procedures on freeze thawed and freeze dried blood there is a need to show that the apparent viability of the RBCs indicates healthy blood cells that are capable of binding oxygen, carrying it and releasing it. The next step in making this determination will be with samples of human blood that have undergone either freeze thawing or freeze drying for use in transfusion to athymic (nude – damaged immune system) rats. Nude rats will be used as the animal model because they are larger than mice making the procedures easier to follow. Transfusion with marked freeze thawed or freeze dried blood with non-radioactive isotope labels and evaluation of the survival of the cells in-vivo will be performed. In addition, blood will be drawn out of the nude rats and will be substituted with human RBCs after each procedure and the oxygen saturation level will be evaluated.

Methods
We will perform the in vivo testing on 10 to 15 nude rats, using the animal model developed in Specific Aim #1. The methods and procedures described there will be repeated, adjusted as necessary according to the results of the animal model testing, this time using RBCs that have been freeze-thawed and freeze-dried.

Interpretation of results
Labeling of the freeze thawed RBCs enables the tracking of the transfused blood to determine survival *in vivo.* In some cases the support systems contained in the body enable the survival of cells that *in vitro* would otherwise not be viable. It is anticipated that the survival rates of the transfused blood will be as high as or higher than those found in the in vitro testing.
In order to determine the effective functioning of the freeze thawed RBCs the athymic rats will be artificially caused to suffer from lowered oxygen levels. It is expected that upon transfusion the oxygen levels will quickly return to normal levels.
Finally, it is expected that the urine tests will not find traces of blood which would indicate poor RBC survival.

SPECIFIC AIM #5: UP-SCALING F THE MTG FREEZING DEVICE AND THAWING TECHNOLOGY FOR USE WITH VOLUMES OF 200 ML TO 600 ML.

Rationale

The MTG freezing technology was developed using a specially designed glass tube that is moved at a constant velocity through the multi-thermal gradients in the freezing blocks. The maximum volume that can be frozen in the device as designed is 8.5 ml. This volume is inadequate for large scale use and, therefore, it is n cessary to up-scale the system to accommodate volumes of 200 – 600 ml (or higher). This up-scaling process will require a combination of engineering and biological R&D.
The up-scaling will require a move from tubes to plastic bags which will necessitate adaptation of the freezing blocks to the new vessel parameters (size, proportions, heat conductivity and movement).

Methods

The concept we are going to apply to large volume freezing is not fundamentally different from our proven experience with small volume freezing.
The basic idea behind the freezing system is to move (pull) a bag of red blood cells (dimensions 300mm x 200mm x 10mm) through a cooled block (the temperature gradient controlled by flow of liquid nitrogen (LN_2) and heating elements) in order to freeze it using the directional freezing technique.

The Blood freezing machine will contain three elements:

a. Freezing chambers / Blocks.
b. Motion System.
c. Computer based control system.

In the sketch (right), a conceptual illustration of the two first elements are shown

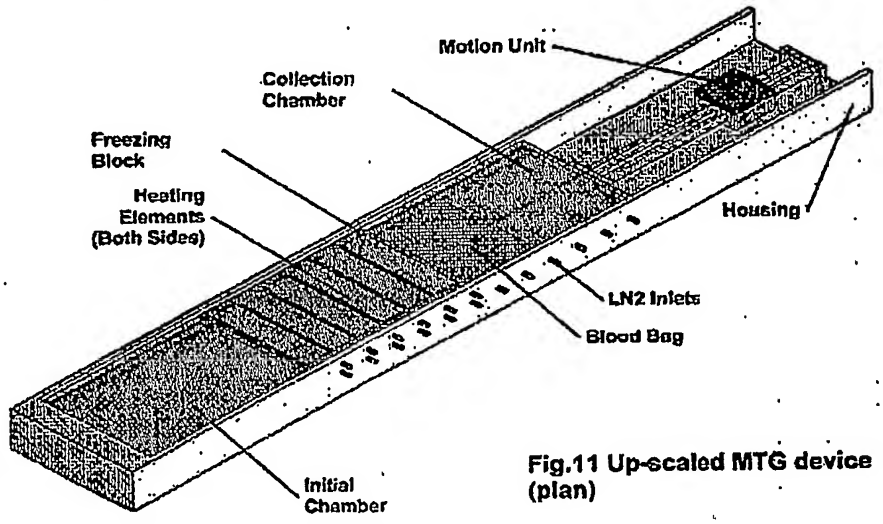
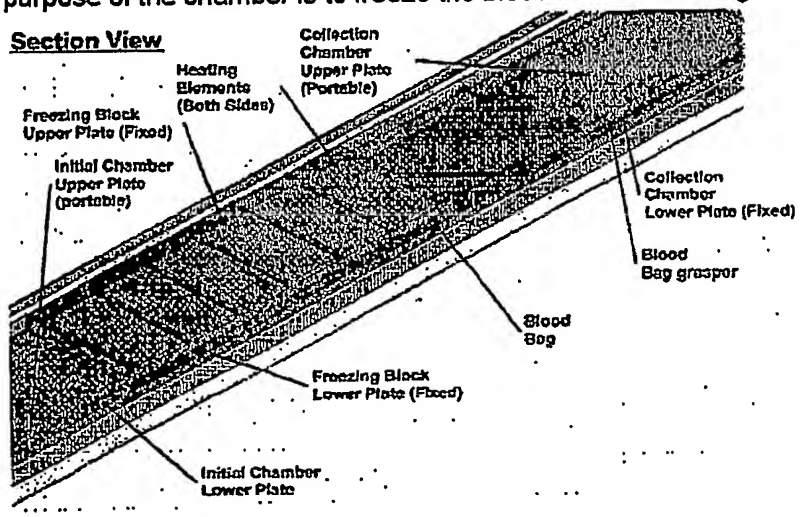


Fig.11 Up-scaled MTG device (plan)

Freezing chambers / Blocks

The first part of the machine will be constructed from three different chambers / blocks.
The first, the "Initial Chamber", is a chamber with portable upper plate. Both, the lower and upper plates will be made from thermal isolating material. The purpose of the chamber is to receive the chilled blood bag, and initiate the freezing process.
The second chamber, the "Freezing Block", will consist of two brass plates with bores for cooling by infusing LN_2 and a series of heating elements (on both sides) to control the temperature gradient. The upper plate will be supported by springs to ensure a full contact between the blood bag and the upper cooling plate. The purpose of the chamber is to freeze the blood in the blood bag.



The third chamber, the "Collection Chamber", will consist of two plates; the upper will be a portable plate, made from thermal isolating material. And the low r plate will be made from brass with bores for cooling by infusing LN_2. The purpose of the chamber is to receive the frozen blood bag, enable the extraction of the blood bag, and enable further cooling to a temperature of -100°C to prevent thermal damage when stored in LN_2

The "Freezing Block" and the "Collection Chamber" will include several thermocouple sensors (not shown in the

sketch) In order to control and maintain the desired temperature gradient along the blocks

Motion System
In order to pull the blood bag through the various chambers, we will use a motion system which will include a motor (not shown in the sketch) and a linear motion slide. The blood bag will be pulled at various controlled velocities, in order to achieve freezing at a predetermined cooling rate.

Computer based control system
In order to control, document and enable the freezing process, we will use an embedded computer system (supplier to be selected after testing different products), which will provide complete control and documentation of the freezing cycle.
The computer will control the LN_2 supply by opening and closing several cryogenic valves. It will also control the pressure in the LN_2 supply tank, the heating elements and it will display all relevant parameters on an integral touch panel display.

Seeding & Thawing Method
The "Seeding" – initiation of the ice crystal formation - will be performed before the initiation of the freezing cycle, external to the freezing machine. The frozen blood bag will be thawed using a regular thawing wat r bath, with water at 37°C. The water temperature will be controlled using a heating element and temperature sensor.

Technological Challenge
The key challenge in designing the up-scaled freezing device is to determine and maintain a desirable temperature pattern. The temperature will be controlled by two forces – cooling by injection of LN_2 and heating by elements. Achieving the correct balance is an engineering challenge to be solved by trial and error and custom part-building.
The other key difficulty is to achieve maximal contact between the blood bag and the cooling plates without damaging the bag. We intend to solve this problem by use of a spring bed; the upper plate will be supported by a sufficient number of springs, which will bear almost all of the upper plate's weight. The remaining force will ensure good contact between the blood bag and the cooling plates. An additional alternative will be to use vertical mountings to hang the blood bag between the plates.

Our goal is to develop an up-scaled freezing device that will operate with the same cooling rate and interface v locity as the device that employed the small glass tubes. We also expect to achieve thawing without recrystallization, thereby providing cell survival results at a level equal to or better than was the case with the small volumes. We may also develop a simple thawing device for these larger volumes that will produce the desired results.

SPECIFIC AIM #6: OPTIMIZE FREEZE-THAWING AND FREEZE DRYING PARAMETERS EMPLOYING THE SCALED UP FREEZING DEVICE
The rationale and methods to be employed in achieving this specific aim are identical to those described in Specific Aim #2. Following the adaptation of the freezing technology to large volumes (200 – 600 ml), our goal is to achieve the same level of cell integrity, cooling rate and interface velocity as were achieved with the small tubes and small volume samples.

Interpretation of results
Specific Aims #6, #7 and #8 will be integrated efforts with results from #6 and #7 providing the feedback necessary for accomplishing SA #8. The significantly larger sample volumes will give rise to a number of issues that must be dealt with in developing the scaled up freezing device. For example, in order to maintain the same cooling rate in large scale, the velocity will probably need to be much slower because the heat needs to pass through a larger mass. We expect to hold environment constant (ratio of RBCs to freezing solution) independent of sample volume, therefore the necessary adjustments will be achieved through engineering and cryo-engineering solutions.

SPECIFIC AIM #7: DETERMINATION OF FUNCTIONALITY IN VITRO OF RBCS BASED ON CELL METABOLISM AND OSMOTIC ROBUSTNESS OF THE CELLS FOLLOWING FREEZE-THAWING EMPLOYING THE SCALED UP FREEZING DEVICE

The rationale and methods to b employed in achieving this specific aim are identical to those described in Specific Aim #3. Following the adaptation of the freezing technology to large volumes (200 – 600 ml) for use in freeze thawing, our goal is to achieve equivalent cell metabolism and osmotic robustness as was the case with the small tubes and sample volumes.

SPECIFIC AIM #8: FULL SCALE UP OF THE MTG FREEZE DRYING TECHNOLOGY TO HIGH TOTAL VOLUMES (E.G. 600 ML) RBCS AND SOLUTION.

Rationale

As with the freeze thawed RBCs, the freeze dried RBCs will require greater volumes for large scale use, th refore we need to adapt the technologies to meet the demands of large volumes (e.g. 200 – 600 ml or higher). In addition, a very large surface area is needed in the freezing and sublimation container that will be used for both processes in order to minimize cell damage. The temperature in the sublimation chamber affects the rate of sublimation and the viability of the cells. The current device achieves -84° in the condenser; the up-scaled device will need to reach -196°. The up-scale will also require a greater vacuum – 10^{-5} Torr as opposed to 10^{-3} at present. This should also speed up the drying process from 3 days at present to approximately 12 hours in the up-scaled device. In order to meet the demands for sublimation of RBCs that have been frozen with the MTG technology at large volumes (e.g. up to 600 ml) we must design a suitable apparatus. These parameters would be suitable also for samples of small volume (e.g. micro liters and a few milliliters) that may benefit from the adapted technology as well.

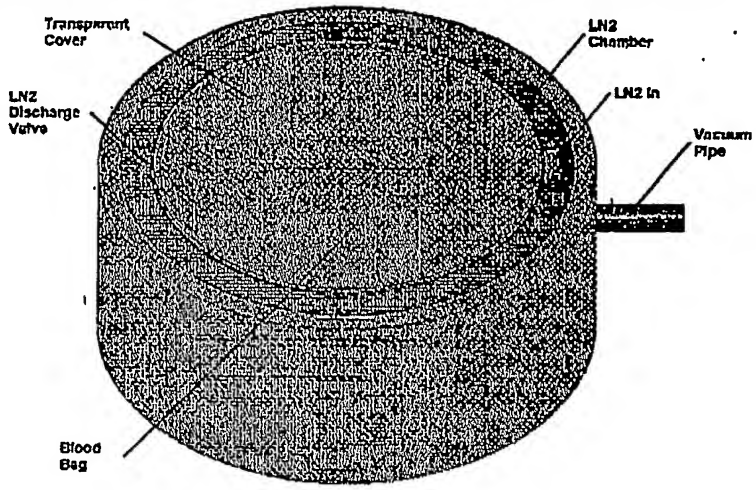


Fig 13: MTG Freeze-drying device (conceptual design)

Methods

The specially designed lyophilizer will be able to dry several blood bags, each containing a volume of 600 ml. In order to achieve a very large temperature difference between the blood bag which is being dried and the condenser, the lyophilizer will be cooled by LN_2 instead of conventional refrigeration methods. Since LN_2 absorbs heat and evaporates during the drying process, a key challenge will be to provide sufficient LN_2 in advance to sustain the entire FD process, or to develop a method of adding LN_2 mid-process.

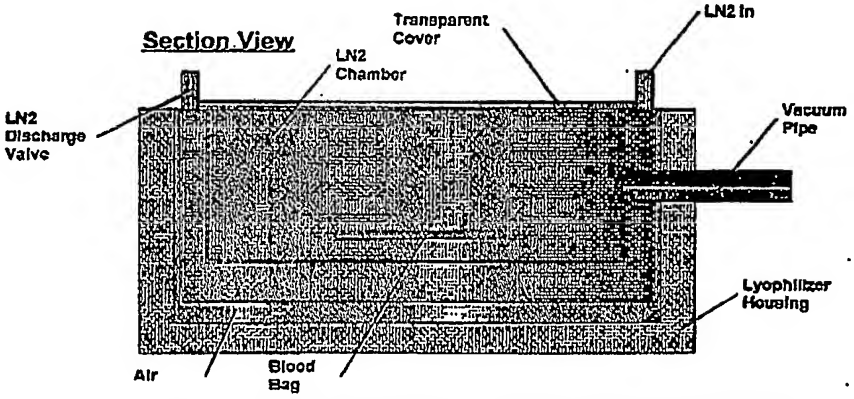


Fig. 14: MTG Lyophilizer – cross section (conceptual design)

The lyophilizer is a cylindrical shaped stainless steel chamber, with an envelope in which the LN_2 is stored. The chamber is isolated from the surroundings by air and a solid housing made from isolating material such as foamed plastic.

To meet the challenge of the high level vacuum we will use two cascaded vacuum pumps; the first will be a turbo pump and the s cond a regular pump. We xpect to achieve a vacuum of 10^{-5} Torr.

The blood bags will be placed on a specially designed structure which will permit the evaporation of all the liquids.

Interpretati n of results
We anticipate the need for integration betw en SA 6, 7 and 8 that will enable us to employ the ongoing feedback from SA 6 and 7 to be incorporated in the development of the new lyophilizer. W believe that we have dedicated sufficient time and resources to the development process of the device as to provide adequate opportunity to perfect the technology.

SPECIFIC AIM #9: TESTING IN VIVO FUNCTIONALITY OF UP-SCALED FREEZE THAWED AND FREEZE DRIED RBCS IN ATHYMIC RATS

Rationale
Once satisfactory results have been achieved *in vitro* regarding the survival (SA #6) and functionality (SA#7) of FT and FD RBCs processed by the up-scaled devices developed in SA#5 and SA#8, we will confirm the results *in vivo* in our animal model before proceeding to human studies. This will also be helpful in obtaining regulatory approval for future clinical trials.

Methods
The methods, experiments and interpretation will be identical to those detailed above in Specific Aims #1 and #4, except that the RBCs to be tested will have been frozen or lyophilized using the scaled-up devices developed in Specific Aims #5 and #8.

F Vertebrate animals

1 Description of Animal Use

We will be using nude (athymic) rats ages 8 – 9 weeks for our *in vivo* experiments with RBCs. The initial work with these animals will be aimed at building an animal model that will be effective in testing RBC survival and functionality post freeze thawing and freeze drying. We anticipate the use of 10 rats for this phase of the project and another 10 to 20 rats during the *in vivo* phase of the project in years 2 and 4.

During the *in vivo* experiments human blood samples, after freeze thawing and freeze drying, will be labeled with isotopes and then transfused into the rats. Then, blood samples will be drawn out from the rat at periodic intervals over approximately 10 days in order to evaluate the amount of the circulating RBCs. The assay will be performed by BioPAL using neutron activation technology.

In order to conduct an evaluation of oxygen saturation level a quantity of blood will be removed from the rats and centrifuged for 10 minutes at 1500g, to separate out the plasma. The plasma only will be re-transfused in order to cause a reduced level of blood oxygen saturation without reducing blood volume. We will then transfuse freeze thawed or freeze dried human RBCs, at the same volume of packed red cells as that which was removed, and then measure the oxygen saturation level of the blood to determine if the level has risen.

Finally, we will perform urine tests to detect blood in the urine which would indicate poor survival of the transfused RBCs.

2 Choice of Animal and Species

The use of nude rats is based chiefly on two factors:

1. Rats are larger than mice enabling easier removal and transfusion of blood and
2. We expect their damaged immune systems to enable them to receive transfusions of human RBCs without rejection.

3 Veterinary Care f Animals

The rats will be cared for by our trained research staff in our specially equipped animal room. There is a veterinary doctor (PI) on site at our facilities and we closely supervise all animals used for research purposes. We will follow all NIH recommended protocols for animal care in conducting this research.

The rats will be held in roomy cages that are filtered as well as being situated in a laminar flow hood. The animal room is temperature and humidity controlled. The cages are regularly cleaned and adequate f od and water are provided on a daily basis.

We buy the rats' food and litter from Harlan Ltd. after they have both been autoclaved. The rats are kept in 12 hours light/dark cycles. Temperature is set to 22C controlled by a thermocouple connected to a heater. When the temperature falls below 21°C, the heater turns on automatically. The ambient humidity is between 40 – 70%.

4 Procedures to Avoid or Minimize Discomfort, Distress or Pain

Blood collections and transfusions with these rats will be done at the jugular vein using an indwelling intravenous catheter (See full description of the surgical procedure that is provided in section D.) To situate the catheter the animals will be anaesthetized using 10 mg/kg xylazine and 90 mg/kg ketamine. The indwelling catheters will be properly bandaged to ensure a maximum of animal comfort.

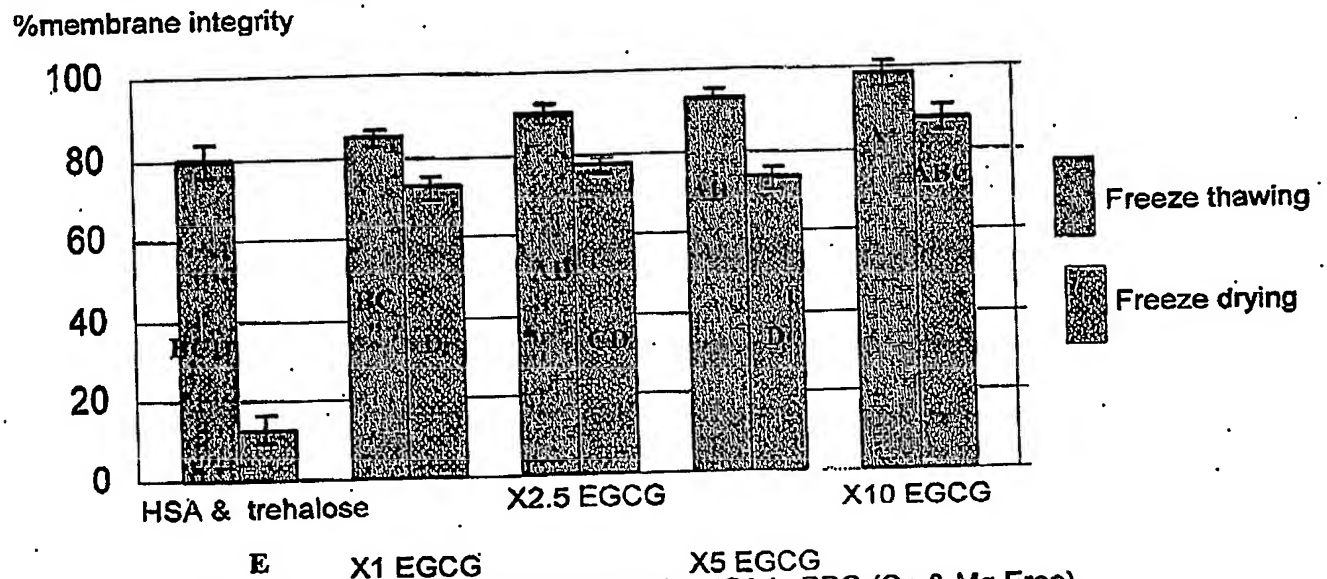
5 Euthanasia

At the culmination of the experiments the rats will be sacrificed with injections of an overdose of Sodium Pentobarbital to the vein. This method is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

G Final Remarks

We have tried adding various antioxidants to the cells being frozen or freeze dried (not shown) and the best results were obtained with EGCG (as detailed above). In fact, we tried EGCG in varying concentration (Fig. 15). The results are depicted in comparison with fresh MNC. As can be seen in Fig. 15, the results for freeze drying at 10X EGCG (515.5 µM), were much improved in comparison with the lower tested concentrations. It is thought by some that at such concentrations the antioxidants may act as oxidants.

Fig. 15 MNC derived from UCB after freeze thawing and freeze drying with different concentration of EGCG



E X1 EGCG X5 EGCG

HSA & Trehalose = 0.1M Trehalose and 12.5%(w/v) HSA in PBC (Ca & Mg Free)
X1 EGCG = 51.5µM EGCG

- All the experiments where done at a cell suspension of 2.5ml. All experiments with EGCG where done with 0.1M trehalose.
- The MNC where obtained by separating on ficol-paque gradient as described in he patent.

We can see how as the EGCG concentration increase so does the membrane integrity.

All the methods described above (including freezing methods and the freeze drying methods) may be applied not only to the cells described, but may also be applicable to other blood products and other cells and groups of cells such as sperm, ova, embryos, etc. They may even be applied to organisms such as microbial cells, bacteria etc.

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Successful Vitrification protocol for cryopreservation of human and mouse embryos

Aims: Vitrification offers a rapid and cost efficient technique for embryo preservation at different stages of their development. This method is based on high cooling and warming rates that are achieved by using low volume techniques (OPS, Cryoloop, EM grids). However, in these techniques contamination may occur due to a direct contact between the embryos and the Liquid Nitrogen (LN). Our aim was to develop a safe and functional technique in which pulled straws are sealed (SPS), to avoid contact between the embryos and LN, and plunged into LN Slush using the VitMaster apparatus (IMT Ltd, Israel).

Methods: Two pronuclei (2PN) mouse embryos were flushed 12 hours after fertilization and left for further development in culture up to 2-cell, 4-8-cell, and early-blastocysts. For vitrification, embryos were exposed to 10% Vitrification Solution (VS) for 1 minute, transferred into 50%VS and immediately thereafter into a final VS (100%VS containing 38% v/v ethylene glycol (EG), 0.5M Trehalose and 6% BSA in PBS) (table 1). Embryos were then loaded into super open pulled straw (SOPS) that were sealed, and vitrified at a rapid cooling rate (CR) of 15,500°C/minute using the VitMaster apparatus. Since blastocysts are most sensitive to decrease in temperature we also tested the effect of slow CR (3,000°C/minute) on early blastocysts. Blastocysts were vitrified in 0.25ml sealed straws in LN. Warming of the embryos was performed by plunging the SPS and the 0.25ml straws into the VitMaster warming chamber at 38°C. Embryos were then immersed in 0.6M Trehalose solution for 4 minutes and transferred though a series of solutions containing decreasing concentrations of trehalose: 0.5M, 0.4M, 0.3M, 0.2M and 0.1M for 2 minutes each. Viability was evaluated by the ability of the embryos to develop into expanded-blastocysts.

In preliminary experiments with human embryos from patients undergoing IVF treatment, thirteen 8-cell embryos and twenty five blastocysts were vitrified. For this purpose, embryos were exposed to VS containing 10% EG, 20% synthetic serum substitute (SSS) and 0.1M Trehalose in PBS, for 2 minutes and then transferred into VS containing 40% EG, 20% SSS and 0.5M Trehalose for 30 seconds. Embryos were loaded into SOPS and plunged into LN-Slush. The day prior to their transfer, embryos were warmed in the VitMaster warming chamber and transferred though a series of solutions containing decreasing concentrations of trehalose, 0.75M, 0.6M, 0.5M, 0.375M, 0.25M and 0.125M for 2 minutes each, washed twice and incubated overnight in culture media. Embryos with the highest morphology rank were selected for transfer.

Results: The results of the mouse embryo experiments are presented in table 1. These results show high survival rates after LN-Slush vitrification of embryos at all stages of their development. Moreover, significantly ($P<0.05$) lower survival rates were observed in blastocysts vitrified by slow CR.

Mouse Embryos

Developmental Stage	N	Cooling Rate [°C/minute]	Final Vitrification Solution	Blastocyst formation/ Re-Expansion*
Blastocyst	99	3,000	87.5%	24% *
Blastocyst	190	15,000	87.5%	57% *
4-8 Cell	188	15,000	87.5%	65%
2 cell	126	15,000	75%	89%
2PN	168	15,000	87.5%	62%

Table 1 – Mouse blastocyst formation / re-expansion rate (Results were normalized from Control).

Human Embryos

Six of the thirteen 8-cell embryos continued to develop after warming (47%) and were transferred into the uterus of 4 patients, resulting in two clinical pregnancies. One of these pregnancies is still ongoing (12 weeks). Fifteen blastocysts out of twenty five (60%), developed into fully expended blastocysts. Thirteen of them were transferred into the uterus of 9 patients. Unfortunately only two chemical pregnancies were achieved.

Conclusion: These results demonstrate that plunging embryos in SPS into LN-Slush permitted increased cooling rate and successful cryopreservation of mouse embryos at all stages in the presence of low concentrations of vitrification solution. We further demonstrated that this technique can be modified for vitrification of human embryos offering a rapid and simple protocol for embryo cryopreservation in IVF treatments.